ARTICLE

Nonmembrane Associated Dolichol in Rat Liver

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The distribution of dolichol in rat liver was studied. Upon high-speed centrifugation, 9% of the total tissue dolichol was recovered in the supernatant. Dolichol was enclosed in vesicles and in lipidic particles which were isolated by gel filtration and density gradient centrifugation. The particles had a diameter of 20 nm and contained dolichol, ubiquinone, cholesterol, phospholipid and some protein. Similar particles were recovered upon incubation of isolated hepatocytes with liposomes containing dolichol. From the lysosomal lumen, lipid particles containing dolichol, ubiquinone, cholesterol and phospholipid, but no protein, were isolated. The diameter of the particles was 20–40 nm with a molecular weight of 130 kDa. Puromycin treatment inhibited protein synthesis, but did not affect dolichol transfer from the endoplasmic reticulum to lysosomes, suggesting that the transfer is not mediated by newly synthesized apoprotein. The results indicate that a sizeable portion of the total cellular dolichol is present in cytoplasm and in lysosomal lumen. Furthermore, dolichol probably participates in the translocation process.

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Ubiquinone and dolichol appear to be located in the central hydrophobic core of membranes (1,2), whereas cholesterol is known to be asymmetrically distributed between the phospholipid fatty acyl groups in the transverse plane of the bilayer (3). The major lipids in the cell are phospholipids, and the types and amounts of phospholipids present can vary somewhat under extreme pathological conditions (4). By comparison, induction of the endoplasmic reticulum or peroxisomes with xenobiotics can increase dolichol concentrations manyfold. This finding raises the possibility that not all dolichol in organelles is membrane associated (5). In addition, biosynthesis of dolichol occurs largely in the endoplasmic reticulum, which necessitates extensive transport to various other organelles (6). Dolichol is also found in the high-speed supernatant fraction, which may imply that dolichol utilizes transport systems involving carrier proteins; such transport processes have been extensively studied for phospholipids (7).

The functions of dolichol in the cell are presently not well understood. Dolichyl-P, which participates in the biosynthesis of glycoproteins (8), is usually present in a small amount and appears to be synthesized by a separate pathway (9,10). In addition to membrane-associated functions, such as regulation of membrane stability, fluidity and permeability, dolichol may be involved in metabolic processes which are not necessarily associated with membranes.

In the present study we have investigated the intracellular distribution of dolichol. The main objective was to find out whether dolichol is mainly associated with membranes or is also found in the cytosol and lumen of some organelles. The functional importance of observed intracellular dolichol distribution was assessed.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 180-200 g were used. In experiments where puromycin (15 mg/100 g body weight) was injected intraperitoneally, the rats weighed 60 g. In the *in vivo* experiments, 0.5 mCi (*RS*)-[5-^{3}H]mevalonolactone (24 Ci/mmol, New England Nuclear, Boston, MA) or 60 μCi [^{14}C]leucine (57 mCi/mmol, Amersham International, Buckinghamshire, England) dissolved in 0.9% NaCl was injected into the portal vein under Mebumal anaesthesia.

Fractionation. For the preparation of microsomal, mitochondrial/lysosomal and supernatant fractions, rat livers were homogenized in 0.25 M sucrose in a glass-Teflon homogenizer with 4 strokes at 440 rpm. The homogenate (20%, w/v) was centrifuged at 310 × *g* for 10 min to remove debris and nuclei, and the mitochondrial fraction was subsequently obtained by centrifuging the resulting supernatant at 3,800 × *g* for 20 min. The pellet thus obtained was washed twice by recentrifugation in 0.25 M sucrose, and thereafter repelleted at 3,800 × *g* for 20 min. This fraction also contained lysosomes and was designated the mitochondrial/lysosomal fraction. The supernatant obtained after centrifugation at 10,000 × *g* for 20 min was recentrifuged in a 50 Ti rotor (Beckman, Palo Alto, CA) at 105,000 × *g* for 1 hr. The resulting microsomal pellet was suspended in 0.25 M sucrose. The high-speed supernatant fractions were prepared by recentrifugation of the postmicrosomal supernatant at 150,000 × *g* for 3 hr in 7-mL centrifuge tubes. The upper 1.5 mL, designated the top-layer supernatant, was removed with a Pasteur pipette. The rest of the supernatant, with the exception of the lower 1 mL (4.5 mL), was analyzed and designated as the middle-layer supernatant.

Lysosomes were isolated on a Metrizamide gradient as described earlier (11). In order to separate membranes and luminal contents, the lysosomes were suspended in distilled water and subjected four times to freeze-thawing, followed by sonication, twice for 1 min, with the fine tip of a Branson sonicator (model S-125) at a setting of 0.5 A. The membranes were subsequently separated by centrifugation at 105,000 × *g* for 2 hr.

To determine crosscontamination in isolated fractions, the various organelles were prepared and analyzed for marker enzymes. The degrees of contamination (1-8%) were similar to those described in detail earlier (12).

Isolation and labeling of hepatocytes. The rat livers were perfused through the portal vein with Hanks' buffer con-

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Abbreviations: EGTA, ethylene-*bis*(oxyethylenenitrilo)tetraacetic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate.

taining 0.5 mM EGTA and 2% albumin, followed by Hanks' buffer containing 0.12% collagenase (Type V, Boehringer Mannheim) and 2 mM $CaCl_2$. The solutions were gassed with 93.5% O_2 and 6.5% CO_2 (carbogen gas) and heated to 37°C prior to use. The perfused livers were shaken in Krebs-Henseleit buffer to disperse the hepatocytes. The cell suspensions were washed once with the same buffer. Cell viability was estimated by incubation with 0.04% trypan blue, and by counting the cells which excluded the dye using a Burker chamber.

Isolated hepatocytes (200×10^6 cells) in 40 mL Krebs-Henseleit buffer were incubated for 60 min at 37°C under a "carbogen" atmosphere with liposomes containing [^{3}H]dolichol-19. The liposomes were prepared by mixing 20 mg egg lecithin (Lipid Products, South Nutfield, England) in chloroform/methanol (2:1, v/v) with 50 nmoles [^{3}H]dolichol-19 (specific activity 5.5×10^6 dpm/nmol) labeled according to Keenan and Kruczek (13). After evaporation of the solvent, 4.5 mL of 0.9% NaCl was added and the suspension was pulse-sonicated for 50 min in an ice-bath under a nitrogen atmosphere. The liposomes containing [^{3}H]dolichol were incubated with hepatocytes at 37°C. The hepatocytes were subsequently sedimented by centrifugation at $50 \times g$ for 5 min and washed five times with 20 mL of 0.9% NaCl. The cells were finally suspended in 20 mL of 0.25 M sucrose. For subfractionation, hepatocytes were sonicated for 30 sec in order to disrupt the plasma membranes, and then subfractionated as described earlier (14).

Gel filtration. In order to isolate the dolichol-containing components in the middle-layer supernatant, 5 mL of this supernatant was subjected to gel filtration on a 1.5×70 cm Bio-Gel A-1.5 m column (Bio Rad, Richmond, CA), 200–400 mesh. The column was eluted with 50 mM Tris-HCl, pH 7.8, at a flow rate of 0.2 mL/min, 2-mL fractions were collected.

Gel filtration on Sephacryl S-200 superfine (Pharmacia, Uppsala, Sweden) was used to estimate the molecular weight of the dolichol-rich vesicles recovered from the lysosomal lumen. The size of the column was 1.6×90 cm, and the elution buffer was 100 mM Tris-HCl, pH 7.0, containing 300 mM NaCl. The flow rate was 0.1 mL/min.

Gradient centrifugation. The peak in the void volume (10 mL) from the Bio-Gel column was mixed with 3.4 g KBr and placed on the bottom of a centrifuge tube. This mixture was overlayed with 28 mL of 0.9% NaCl and centrifuged in a VTi-50 vertical rotor at $203,000 \times g$ for 3 hr. Fractions of 1.3 mL were then collected from the tube.

Flotation of lysosomal contents was carried out in a vertical VTi-65 rotor by mixing 3 mg of the content protein with solid KBr and water to adjust the density to 1.3 g/mL. The fraction was overlayed with 1.1 mL 0.9% NaCl and centrifuged at $203,000 \times g$ for 5 hr. The tubes were punctured at the bottom and 10 fractions collected. The last fraction was designated the uppermost fraction. A number of lysosomal enzyme activities were assayed in the lysosomal fractions, and all of these activities were absent in the uppermost fraction.

Lipid analysis. Dolichol and cholesterol were assayed by HPLC, as described earlier (15). The lipid extract was subjected to alkaline hydrolysis and applied to a C18 Sep-Pak column (Waters, Milford, MA). Cholesterol was collected in the methanol phase and dolichol was subsequently eluted with hexane. HPLC was performed using a Hewlett-Packard (Palo Alto, CA) Hypersil ODS (C18) 3μ column. For analysis of dolichol, a linear gradient from the initial methanol/2-propanol/water (60:40:5, v/v/v) in pump system A to methanol/2-propanol/hexane (60:40:20, v/v/v) in pump system B was employed. The flow rate was 2 mL/min and the program time was 25 min. The absorbance was recorded at 210 nm.

For analysis of cholesterol, a linear gradient from the initial (A) methanol/water (9:1, v/v) to (B) methanol/2-propanol (8:2, v/v) was used. The flow rate was 2 mL/min and the program time 25 min; the eluate was monitored at 210 nm. Dolichol (C23) and ergosterol were added as internal standards prior to alkaline hydrolysis.

Ubiquinone is partially degraded by alkaline hydrolysis and therefore the extraction was done with chloroform/methanol/water (1:1:0.3, v/v/v) as described earlier (16). In HPLC the same linear gradient was used as for the analysis of cholesterol. Elution was monitored at 275 nm. Ubiquinone-6 was added as internal standard before extraction. Phospholipids were measured as described earlier (17).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Aliquots of fractions were dialyzed against distilled water and concentrated by lyophilization. Electrophoresis was then performed in 1.5-mm vertical slab gels with 10–15% polyacrylamide, according to Laemmli (18).

Electron microscopy. The samples were fixed in 3% glutaraldehyde in 0.2 M sodium cacodylate-HCl with 0.25 M sucrose, pH 7.2, at 4°C overnight. Following postfixation in 1% osmium tetroxide in 0.15 M sodium cacodylate-HCl, pH 7.2, for 90 min at 20°C, the samples were dehydrated and embedded in epoxy resin.

For negative staining a drop of the specimen was placed on the copper grid covered by a thin film of Formvar, which was stabilized with a 40 Å coating of carbon. After 15 min at 20°C, the fluid was absorbed with filter paper and one drop of 2% uranyl acetate was added.

Immunoprecipitation. For the isolation of plasma proteins, microsomes were solubilized with sodium deoxycholate at a final concentration of 0.5% and mixed with protein-A-Sepharose CL-4B (Pharmacia). After centrifugation, aliquots of the supernatant were mixed with excess antibody against rat albumin, fibrinogen and transferrin. After 1 hr at room temperature, incubation was continued at 4°C overnight. The samples were chromatographed on a protein-A-Sepharose column.

Protein precipitation. Protein content was measured according to Lowry *et al.* (19) using bovine serum albumin as standard.

RESULTS

Isolation and metabolism of dolichol. After centrifugation at high g force of the postmicrosomal supernatant, a white, milky top-layer was obtained. This "top-layer" was removed while the remainder of the supernatant was designated as "middle-layer". Most of the dolichol and dolichyl ester was recovered in the top-layer (Table 1). Approximately 20% of the total free dolichol was present in the middle-layer, and only a minor portion was present in esterified form.

Upon injection of [^{3}H]mevalonate into the portal vein of rats, the initial rate of incorporation of label into

TABLE 1

Distributions of Dolichol and Dolichyl Ester in Rat Liver Cytosol

Fraction	Dolichol (free alcohol)	Dolichyl ester
	(μg/g liver)[a]	
Top-layer	0.8 ± 0.1	1.4 ± 0.2
Middle-layer	0.5 ± 0.01	0.04 ± 0.005
Total supernatant	1.3 ± 0.2	1.4 ± 0.2
Homogenate	26.2 ± 3.3	2.4 ± 0.3

[a]The values are the means ± SE of six experiments.

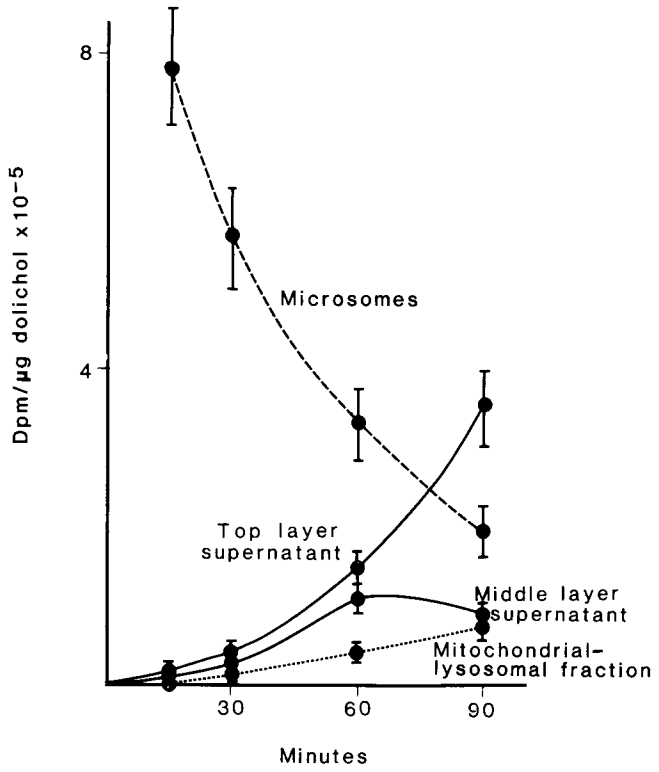

FIG. 1. Incorporation of radioactivity into dolichol of various hepatic subcellular fractions after injection of [^{3}H]mevalonate into the portal vein of rats. At indicated times, the livers were removed for subcellular fractionation and for the isolation of dolichol as described in Materials and Methods.

isolated microsomes was high and then decreased rapidly, presumably due to transport of newly synthesized lipid to other cellular compartments, catabolism, and/or production of other metabolites (Fig. 1). During the first 10 min after injection, no labeled dolichol was detected in the cytosol, but later on labeling in both the middle- and the top-layer increased. In the middle-layer maximal incorporation was attained at 60 min after injection, whereas in the top-layer the specific radioactivity in dolichol continued to increase for at least 90 min. Labeling of dolichol in the mitochondrial/lysosomal fraction was low initially, but subsequently increased slowly.

Size distribution of particles containing dolichol. Millipore filtration was routinely used to estimate the size

TABLE 2

Millipore Filtration of the Supernatant Fraction After *in vivo* Labeling with [^{3}H]Mevalonate[a]

Pore size (μm)	Dolichol (% of total in filtrate)[b]	
	Top-layer	Middle-layer
8.0	81 ± 10	96 ± 12
3.0	76 ± 8	92 ± 12
0.8	23 ± 4	86 ± 10
0.3	21 ± 3	81 ± 10
0.1	24 ± 4	76 ± 8
0.05	18 ± 3	63 ± 8
0.025	22 ± 4	52 ± 6

[a]The supernatant fractions were applied to the filter and washed with 0.25 M sucrose.

[b]The numbers denote the percent of the original dolichol recovered in the filtrate. The values are the means ± SE of three experiments.

of particles in both layers of the high-speed supernatant fraction (Table 2). When the top-layer was passed through filters with decreasing pore size and the amounts of dolichol in the different filtrates were measured, we found that at a pore size of 0.8 μm only 23% of the dolichol appeared in the filtrate. By comparison, as much as half the dolichol was recovered in the filtrate of the middle-layer, even with a pore size of 0.025 μm.

These filtration experiments demonstrated that the dolichol in the top-layer is associated with structures larger than microsomal vesicles. Electron microscopy revealed the existence of membrane-bound vesicles of various sizes, most ranging from 1.5–2.5 μm (Fig. 2). Most of the vesicles contained material that was stained heavily with osmium and apparently was lipid. Since this fraction contained both membranous and nonmembranous materials, it was not further characterized.

Properties of the middle-layer dolichol in the high-speed supernatant fraction. The dolichol present in the middle-layer in nonmembrane-bound form was isolated and further characterized. When the layer was subjected to gel filtration on a Bio Gel column, most of the dolichol-containing fraction was excluded (Fig. 3A). The excluded material was then subjected to centrifugation on a KBr gradient (Fig. 3B), and we found that most dolichol-containing material equilibrated at a density of approximately 1.05 g/mL. The fraction isolated by gradient centrifugation (Table 3) contained mostly phospholipid, some cholesterol (about 20%) and dolichol at about 0.2% of the total lipid content.

SDS-PAGE analysis indicated substantial enrichment of proteins from the middle-layer upon chromatography on Bio Gel and further enrichment on the KBr gradient (Fig. 4). In the fraction isolated by gradient centrifugation, the five major protein bands had molecular weights of approximately 30, 65, 150, 250 and 300 kDa.

The fraction isolated by gradient centrifugation was also characterized by electron microscopy using negative staining (Fig. 5). Particles with a diameter of about 20 nm were visible and apparently were of the lipoprotein type.

In vitro *lipid transfer.* Previous experiments had demonstrated that isolated hepatocytes take up liposomal vesicles that contain dolichol (20). The uptake apparently does not involve the LDL (low density lipoprotein) receptor or endocytotic vesicles. When the hepatocytes were subfractionated in the same manner as hepatic

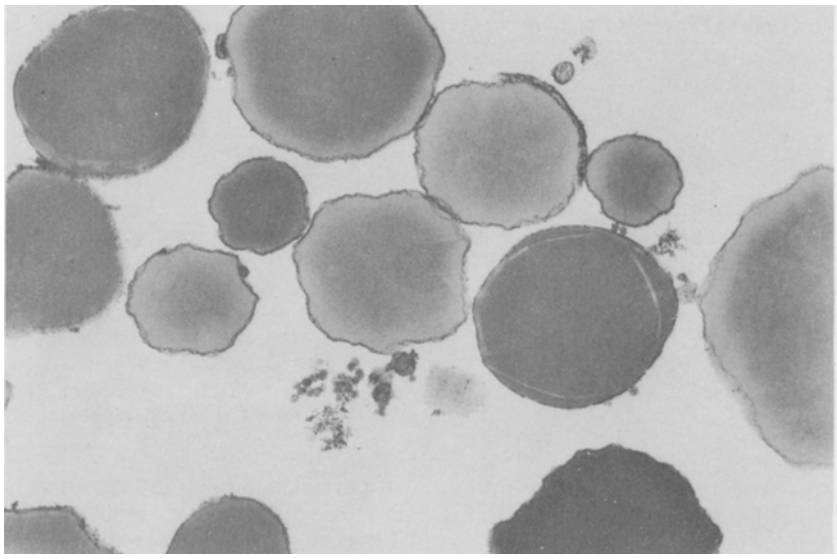

FIG. 2. Electron micrograph of the vesicles in the top-layer supernatant. Lipid-filled vesicles with a diameter of 1.5–2.5 μm are seen. Magnification × 25,600.

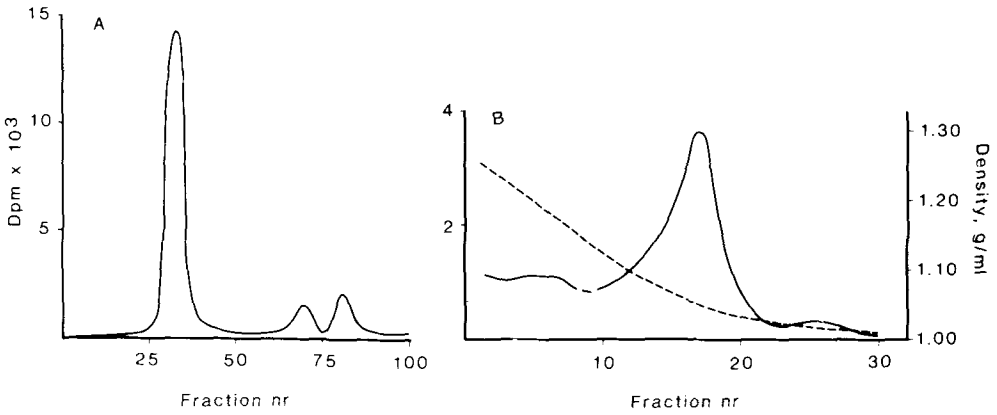

FIG. 3. Purification of the dolichol-containing fraction from the middle-layer supernatant. A. The middle-layer supernatant was prepared 60 min after the injection of [^{3}H]mevalonate, 5 mL was placed onto a Bio-Gel A-1.5 m column (1.5 × 70 cm), and fractions (2 mL) were eluted with 50 mM Tris-HCl, pH 7.8, at a flow rate of 0.2 mL/min. Dolichol was isolated from the eluted fractions by HPLC and analyzed for radioactivity. B. The dolichol-rich fraction of the void volume after gel filtration was subjected to centrifugation on a KBr gradient. After centrifugation, the contents of the centrifuge tubes were divided into 1.3 mL fractions and analyzed for density. Dolichol was isolated and its radioactivity determined.

tissue, a continuously increasing level of radioactivity was associated both with the middle- and the top-layers (Fig. 6). In this way it was possible to obtain dolichol-containing cytoplasmic structures with a high level of radioactive labeling.

The middle-layer first was subjected to gel filtration followed by gradient centrifugation in order to purify the dolichol-rich fraction. This purified fraction was then used in lipid transfer studies. Increasing amounts of the purified fraction resulted in a linear increase in transfer of labeled dolichol into the mitochondrial/lysosomal fraction (Fig. 7A). An excess of cytoplasmic [^{3}H]dolichol in the incubation medium resulted in a time-dependent dolichol transfer to both the mitochondria and the microsomes (Fig. 7B and 7C).

Lysosomal content. Lysosomes contain 20 times more dolichol (4.5 μg/mg protein) than do microsomes. Upon sonication or osmotic treatment, 90% of the lysosomal dolichol was solubilized without solubilizing the lysosomal membrane. The dolichol removed from the lumen of lysosomes was mixed with KBr and centrifuged in a self-generating gradient using a vertical rotor. The top fraction, with a density of less than 1.20 g/mL, was examined further using negative staining electron microscopy.

TABLE 3

Lipid Composition of the Dolichol Containing Fraction Purified from the Middle-Layer Supernatant by Gel Filtration and Density Gradient

Lipid	Amount (μg)[a]
Phospholipid	52.3 ± 5.9
Dolichol	0.18 ± 0.03
Cholesterol	13.8 ± 1.5
Ubiquinone	0.27 ± 0.04

[a]The values represent the lipid contents in the fraction obtained from 1 g liver. The values are the means ± SE of six experiments.

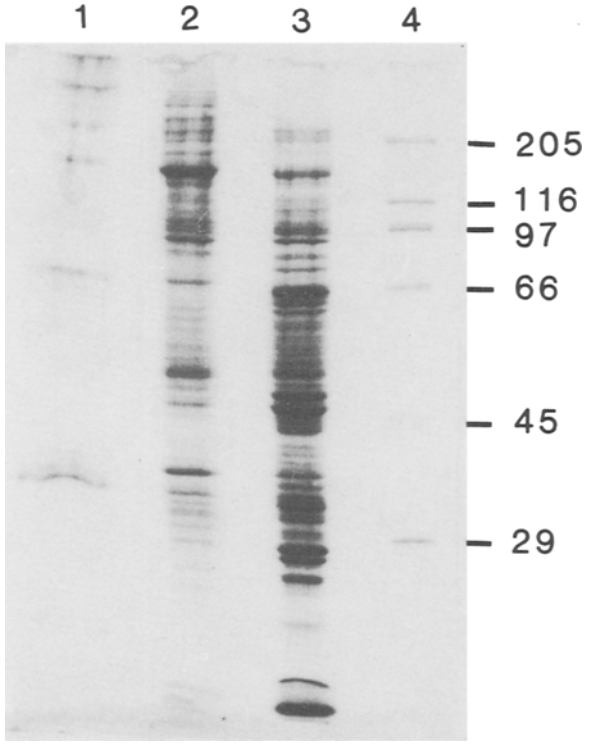

FIG. 4. SDS-polyacrylamide gel electrophoresis of the proteins in the dolichol-containing fraction after Bio-Gel chromatography and density gradient centrifugation (lane 1), dolichol-containing fraction after Bio-Gel chromatography only (lane 2) and middle-layer supernatant (lane 3). Molecular weight standards consisted of myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) (lane 4).

FIG. 5. Negatively stained preparation of the purified dolichol-containing particles. The diameter of the particles is 20 nm. Magnification × 240,000.

Round particles with a diameter of 20–40 nm and homogenous, non-staining inner contents were seen (Fig. 8). In contrast to many types of negatively stained lipoprotein particles, no grooves containing contrast solution were found.

The fraction of lysosomal contents obtained by flotation consisted mainly of lipid (Table 4). The total lysosomal contents contained phospholipid, cholesterol, dolichol and ubiquinone. About half the total phospholipid placed on the gradient was recovered in the uppermost fraction, while almost all the cholesterol, dolichol and ubiquinone of the lysosomal contents flotated upon centrifugation in this system. No protein was detectable by chemical assay, and virtually no protein bands appeared when the uppermost fraction was analyzed by SDS-gel electrophoresis (not shown).

The content of the uppermost fraction was analyzed on a Sephacryl S-200 superfine column and lipid was measured in the eluted fractions. Dolichol eluted exclusively at an apparent molecular weight of about 130 kDa. The other lipids of the uppermost fraction also appeared at this location.

Puromycin treatment. Dolichol is present in the lumen of lysosomes in nonmembrane-associated form. One explanation for this may be that the lipid synthesized in the endoplasmic reticulum is transported *via* the luminal pathway to the lysosomes through the Golgi system. The transport may or may not involve carrier proteins.

Puromycin is known to inhibit protein synthesis both *in vivo* and *in vitro* by terminating polypeptide elongation

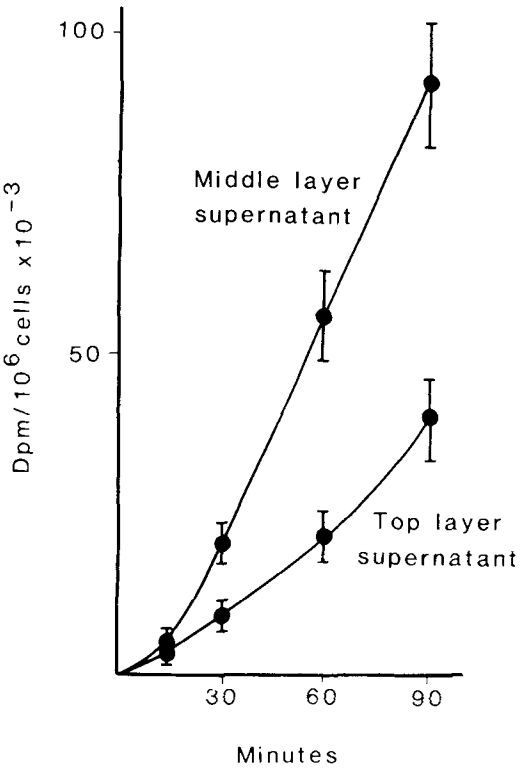

FIG. 6. Recovery of liposomal [^{3}H]dolichol-19 in the supernatant fractions of hepatocytes. Liposomes containing [^{3}H]dolichol-19 were incubated with hepatocytes. At indicated times the reaction was stopped by adding 0.9% NaCl and the hepatocytes were sedimented by centrifugation. The washing procedure was repeated five times. High-speed supernatant fractions were prepared from these hepatocytes, and dolichol was isolated and radioactivity counted.

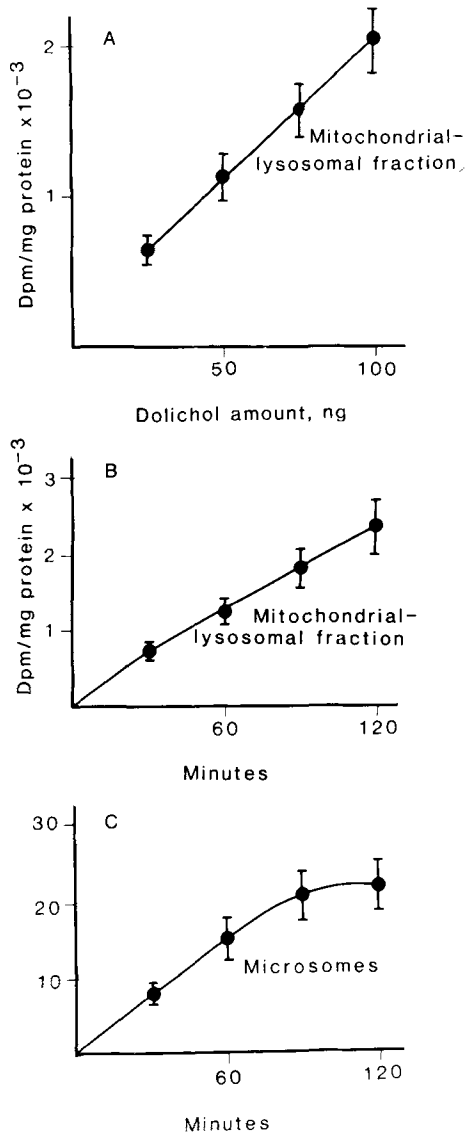

FIG. 7. Transfer of dolichol from purified vesicles to mitochondrial/lysosomal and microsomal fractions. The middle-layer supernatant fraction from hepatocytes labeled with liposomal [^{3}H]dolichol-19 was purified using gel filtration and density gradient centrifugation. The purified fractions were incubated with isolated mitochondria/lysosomes and microsomes. A. Fractions containing 25–100 ng dolichol were incubated for 60 min at 37°C with 12.5 mg mitochondrial/lysosomal protein in the presence of 50 mM Tris-HCl, pH 7.5. B. Fractions containing 50 ng dolichol were incubated for various times at 37°C with 12.5 mg mitochondrial/lysosomal protein. C. As in B, except that the incubation mixture contained 12.5 mg microsomal protein.

at the ribosomal level. Such treatment is useful in the study of export proteins, because most have short half-lives in the endoplasmic membrane system. Under the same conditions, most metabolic processes mediated by complex enzyme systems are unaffected by puromycin, because the majority of the constitutive enzymes have half-lives in the range of days (21).

The effectiveness of the inhibition of protein synthesis by puromycin under our conditions was tested by analyzing [^{14}C]leucine incorporation into newly synthesized plasma proteins (Table 5). Using immunoprecipitation and isolation on protein-A-Sepharose, highly labeled, newly synthesized albumin, fibrinogen and transferrin were separated from the solubilized microsomal fraction. Treatments of rats with puromycin 2 hr prior to removal of the liver resulted in complete inhibition of the biosynthesis of all three of these plasma proteins. Thus, the puromycin treatment completely inhibited protein synthesis associated with the endoplasmic reticulum.

Microsomal dolichol exhibits a high initial level of labeling when [^{3}H]mevalonate is used as precursor *in vivo*, because dolichol is synthesized in the endoplasmic reticulum (Fig. 9). The rapid decay of this radioactivity indicates that the transport to other locations may occur. Simultaneously, the lysosomal pool of dolichol shows increasing labeling, which may indicate that at least a portion of the newly synthesized dolichol is transported to the lysosomes.

Puromycin treatment moderately inhibited the labeling of microsomal dolichol by [^{3}H]mevalonate. In spite of this inhibition, however, the time-course of the decrease in microsomal radioactivity was very similar to that of the control. Puromycin treatment caused only a slight inhibition in the labeling of lysosomal dolichol. The specific radioactivity in both control and treated lysosomes in-

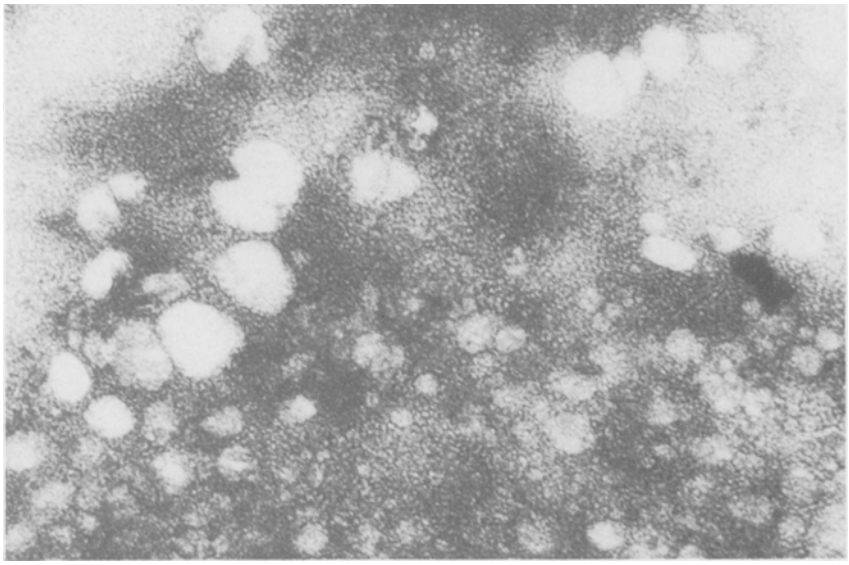

FIG. 8. Electron micrograph of the uppermost fraction obtained by flotation. The lysosomal content was flotated on a KBr gradient and the uppermost fraction examined by negative staining. The size of the lipidic particles is approximately 20–40 nm. Magnification × 160,000.

TABLE 4

Chemical Composition of the Uppermost Fraction Obtained by Flotation of Lysosomal Contents on a Density Gradient[a]

Lipid	Uppermost fraction (μg)[b]	Total content (μg)[b]
Phospholipid	61.2 ± 7.1	111.3 ± 15.4
Cholesterol	8.2 ± 1.1	9.6 ± 0.8
Dolichol	3.7 ± 0.3	4.2 ± 0.5
Ubiquinone	1.4 ± 0.2	1.8 ± 0.3
Protein	ND[c]	540 ± 45

[a]The preparation of lysosomal contents, flotation and measurements of various lipids are described in Materials and Methods.

[b]The values represent the lipid contents obtained from 1 mg lysosomal protein. The values are the means ± SE of six experiments.

[c]ND, nondetectable.

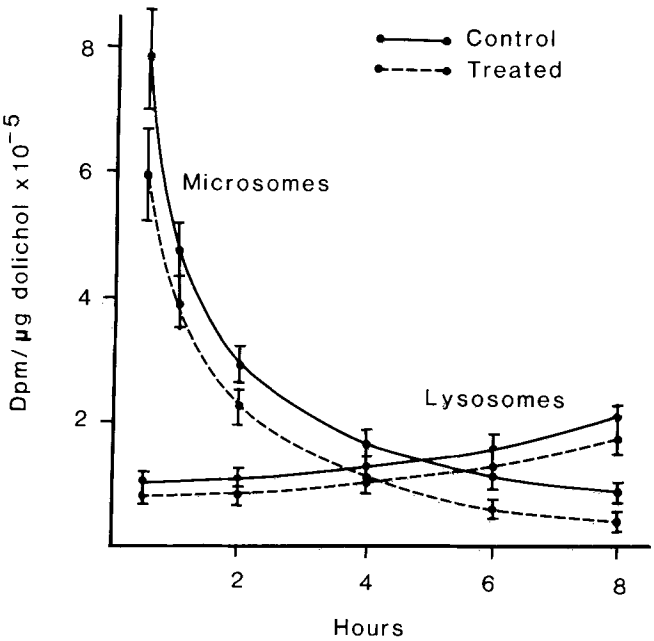

FIG. 9. Appearance of dolichol in microsomes and lysosomes after treatment of rats with puromycin. Puromycin was injected 2 hr and 5 min before the injection of [^{3}H]mevalonate. Puromycin treatment and preparation of the fractions were as described in Materials and Methods. The vertical bars represent the means ± SE of six experiments.

TABLE 5

Inhibition by Puromycin of Plasma Protein Synthesis in Rat Liver *in vivo*[a]

Animals	[^{14}C]Leucine incorporation into		
	Albumin	Fibrinogen	Transferrin[b]
	(Dpm/mg protein)[c]		
Control	4400 ± 422	738 ± 103	1830 ± 238
Puromycin treated	71 ± 7	19 ± 3	72 ± 11

[a]Rats were treated with puromycin (15 mg/100 g body weight) 2 hr before injection of 60 μCi [^{14}C]leucine into the portal vein.

[b]Precipitation with antibody is described in Materials and Methods.

[c]The values are the means ± SE of six experiments.

creased continuously with time. The above experiments demonstrate that puromycin abolishes synthesis of luminal export proteins, but does not hinder the transport of dolichol from its site of synthesis to lysosomes.

DISCUSSION

Most cellular lipids are present in membrane-bound form. Upon subcellular fractionation it has also become apparent that all organelles isolated from rat liver contain dolichol (22). Based on model membrane studies, it has been suggested that dolichol is typically located in the hydrophobic core of the phospholipid bilayers (1,23). This, of course, limits the amount of dolichol which membranes can accommodate. By comparison, the amount of dolichol in subcellular organelles varies greatly, *i.e.*, lysosomes contain 30 times more dolichol than do microsomes. For example, the human pituitary gland contains 7 mg dolichol/g wet weight, which is more than the total content of phospholipid (6 mg/g) (24). It would appear unlikely that all dolichol in this tissue would be membrane associated.

In the present study we found that dolichol can be recovered both in the high-speed supernatant fraction and from the lysosomal lumen (9% and 31% of the total liver dolichol, respectively), which raises questions concerning the physical association of this lipid at these locations. In the supernatant fraction dolichol is concentrated in the fatty layer, as well as in particles of the middle-layer. Electron microscopy revealed that the supernatant dolichol is enclosed in vesicles. Whether this is a storage form, as has been described for secretory proteins (25), or serves a mode of lipid translocation from the site of synthesis to the final site of product use (26) remains to be investigated. Lipid vesicles can be produced by endocytotic processes, a mode that is utilized for uptake of low density lipoproteins (27). However, such a mode is less likely for the formation of dolichol-containing vesicles, because of the labeling patterns observed in the *in vivo* experiments.

Dolichol in the supernatant is also associated with particles 20 nm in size. This particle contains various lipids and proteins, and one or several of these proteins may serve a carrier function in dolichol transport. It cannot be ruled out that the large size of these dolichol particles is the result of secondary aggregation of subunits that could occur during the isolation procedure. However, these particles exhibit chromatographic properties, densities, and compositions, which makes these particles clearly distinct. A transport function of these particles is indicated by the observed labeling patterns, as an increase in radioactivity in these particles occurs when labeling in the microsomes is lost.

When hepatocytes were incubated with dolichol-containing liposomes, dolichol appeared preferentially in the cytoplasm, and the properties of these particles were similar to those that were recovered from rat liver after mevalonate labeling *in vivo*. The close similarity of the middle-layer particles in the *in vitro* and *in vivo* experiments was demonstrated by assessing density, particle size, electron microscopic appearance, and composition. However, *in vivo* labeling occurred at a higher rate in the top-layer vesicles than in the middle-layer supernatant. These differences are likely to reflect the different processes that are involved. One is the transfer of liposomal, labeled dolichol and the other is the *de novo* dolichol synthesis. Dolichol in the cytoplasmic form was transferred to mitochondria and microsomes upon *in vitro* incubation. It appears possible that dolichol is transferred from the site of synthesis *via* the cytoplasm to its final site of cellular use. This type of transport would be reminiscent of that described for phospholipids, the latter being *de novo* synthesized in the endoplasmic reticulum and distributed then through the cytoplasm to various intracellular membranes. Cytoplasmic transport of dolichol is consistent with the model developed by van Dessel *et al.* (28) based on binding studies.

Most dolichol in lysosomes is present in nonmembranous form, together with other lipids and without association to proteins. Labeling studies had previously established that after synthesis in the ER, dolichol is transported to the lysosomes (29). There are several routes for this transport process to occur. One possibility is transport in the ER lumen to the Golgi system, followed by translocation to the newly synthesized primary lysosomes, a route resembling the pathway described for lysosomal enzymes (30). Puromycin treatment inhibited protein synthesis completely without inhibiting dolichol transport, indicating lack of involvement of a luminal carrier protein. This type of lipid transport is not unexpected, because there is evidence that lipoproteins are first assembled in the Golgi system (31). Since primary lysosomes are the product of "exocytosis" of Golgi membranes, a transfer of dolichol in membrane-bound form is also possible. However, the predominantly free occurrence of this lipid in the lysosomal lumen argues against this type of transfer. The transfer of dolichol between ER and lysosomes could also involve a cytoplasmic route, because the particles in the middle-layer exhibited an intermediate labeling pattern.

Several explanations are possible for the presence of free dolichol in the lysosomal lumen. Consistent with their major function, lysosomes may be involved in the modification or breakdown of dolichol. Accumulation in the lumen could also be a step towards a storage process, which could be of a temporary nature. Not unlike cholesterol, newly synthesized dolichol also may be translocated to the bile (32). Some of the dolichol in the lysosomes may originate from the blood by endocytosis. However, this can only account for a minor portion of the total lysosomal dolichol. Finally, one cannot exclude the possibility that there are several dolichol pools in the cell of which the lysosomal pool represents a separate entity.

The extensive transport of dolichol within the cell appears to necessitate the presence of a nonmembranous form at various locations. The lipid is transported to the blood and also to the bile (33,34). In peroxisomes, local synthesis of dolichol is known to occur, and a rapid decay after *in vivo* labeling indicates that rapid transport from this pool takes place (35). In analogy to the enzymes catalyzing its biosynthesis, dolichol may be located in the lumen of the peroxisomes, as it is in lysosomes. The large amount of dolichol in endocrine human tissues suggests nonmembranous storage at this site.

Luminal transport processes may not only take place for dolichol, but also for its breakdown products. Dolichol metabolites have been suggested to be present in bile and at other locations (34). It is possible that these metabolites are simply breakdown products. Alternatively, however, they may represent active substances, including various hormones modulating function. The high turnover of dolichol in the liver, together with the lack of specific and identified end-products, would point in this direction.

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Compartmental Study of Rat Renal Phospholipid Metabolism

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Phospholipid content and metabolism were studied in rat renal papillary, medullary and cortical slices. The highest concentration of phospholipids was found in cortex and the lowest in papilla samples (ratio cortex/medulla, 1.3; cortex/papilla, 3.7). The profile of the various phospholipids was different depending on the zone. The most important difference was the relative concentrations of sphingomyelin (Cer*P*Cho) and phosphatidylinositol (PtdIns) with ratios for PtdIns/Cer*P*Cho of 5.0, 3.3 and 2.5 in papilla, medulla, and cortex, respectively. In the three zones, PtdIns showed the highest specific activity for [2-^{14}C]glycerol and [1-^{14}C]arachidonic acid incorporation. By contrast, a higher amount of [1-^{14}C]palmitic acid was incorporated into phosphatidylcholine than into any other phospholipid. The various radioactive precursors were only poorly incorporated into phosphatidylethanolamine. No radioactivity was associated with phosphatidylserine. The papilla possesses the most active phospholipid metabolism of all the pathways studied.
Lipids 27, 10-14 (1992).

The kidney can be divided into three zones, the papilla, the medulla and the cortex, each having different functions. The zones are composed of various segments of nephron. The cellular composition of nephron has been established by the Renal Commission of the Union of Physiological Sciences (1).

The involvement of lipids in cell membrane structure and function has been well accepted (2). In the kidney, phospholipids comprise more than half of the total lipid pool (3,4).

There is substantial evidence that specific phospholipids can modulate enzyme activities (5,6). The relative distribution and composition of phospholipids in different cellular membranes also influence membrane fluidity (7,8); therefore, phospholipids would be expected to be involved in the regulation of kidney function.

The purpose of our study was to determine whether there are differences in phospholipid metabolism in the three zones of rat kidney. Specifically, we examined endogenous phospholipid contents and profiles, phospholipid *de novo* synthesis, and phospholipid fatty acid turnover in rat renal papilla, medulla and cortex.

MATERIALS AND METHODS

[1-^{14}C]Arachidonic acid (spec. act., 52.8 mCi/mmol), [2-^{14}C]glycerol (spec. act., 14.1 mCi/mmol) and [1-^{14}C]-palmitic acid (spec. act., 53.0 mCi/mmol) were obtained from New England Nuclear (Boston, MA). Silica Gel G plates (0.25 mm thick) and Fiske-Subbarow reagent were purchased from Sigma Chemical Co. (St. Louis, MO). X-Ray film for autoradiography was obtained from Eastman Kodak Co. (Rochester, NY).

Preparation of tissue slices. After decapitation of male Wistar rats (250-280 g), both kidneys were removed and maintained in ice-cold Krebs solution. Each kidney was cut in half through the pelvis along its longitudinal axis, and the papilla (inner medulla), medulla (outer medulla) and cortex were isolated by scissor and scalpel dissection. The renal papilla, medulla and cortex were sliced (*ca.* 0.5 mm thick) using a Stadie-Riggs microtome. The tissue slices were collected in Krebs Ringer bicarbonate buffer containing 5.5 mM glucose. The medium was gassed with 95% O_2/5% CO_2.

Incorporation of radioactive precursors. For each experiment, 5 mg of tissue slices were collected in 0.5 mL of Krebs Ringer bicarbonate buffer containing 5.5 mM glucose. The medium was gassed with 95% O_2/5% CO_2.

Samples were incubated at 37°C with 0.12 μCi of [1-^{14}C]arachidonic acid, 0.12 μCi of [1-^{14}C]glycerol, or 0.12 μCi of [1-^{14}C]palmitic acid in a metabolic shaking bath under an atmosphere of 95% O_2/5% CO_2 for different periods of time. Time course experiments were performed, but the results in the Figures correspond to the time of the steady-state equilibrium (60 min for arachidonic and palmitic acid, 90 min for glycerol).

Extraction and separation of lipids. Incubations were stopped on ice, and samples were washed three times by centrifugation in an ice-cold incubation medium. After the addition of four parts of chloroform/methanol (1:2, v/v) (9), samples were homogenized in glass tubes with a Teflon pestle at 3000-5000 rpm. Phases were separated by adding one part of chloroform and one part of water (10).

The lower chloroform phase was removed. The inorganic phase was washed twice with chloroform, and the organic phases were pooled and dried under a stream of nitrogen at 25°C. This extraction procedure ensures 80-85% recovery of lipids. The extracts were redissolved in chloroform and applied onto precoated thin-layer plates (0.25 mm thick). Lipids were separated by one-dimensional, two-solvent system thin-layer chromatography. The first solvent system used was a mixture of chloroform/methanol/acetic acid/water (40:10:10:1, by vol). The plates were dried and developed to a level 0.6 cm below the first solvent front in a solvent system consisting of chloroform/methanol/acetic acid/water (120:46:19:3, by vol). The R_f values in this system were 0.20, 0.30, 0.47, 0.55 and 0.70 for sphingomyelin (Cer*P*Cho), phosphatidylcholine (PtdCho), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn), respectively; the free fatty acids migrated in the solvent front (11). Lipid fractions were detected with I_2 vapor or by autoradiography. Zones of the thin-layer chromatographic plates corresponding to the various phospholipid fractions were

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Abbreviations: AA, arachidonic acid; C, cortex; Cer*P*Cho, sphingomyelin; Gro, glycerol; M, medulla; P, papilla; PL, phospholipid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.

scraped off and added to 6.0 mL of toluene/omnifluor (0.4%). Phospholipids were quantified in a liquid scintillation counter. Counting efficiency for ^{14}C was 75%.

Endogenous phospholipid determination. For the quantitation of the phospholipids, specific areas of the plates were scraped off and digested with 70% perchloric acid in the presence of ammonium molybdate (0.5%). The resulting inorganic phosphate was assayed with Fiske-Subbarow reagent (12). Recovery of added PtdCho before extraction was 85%.

All results were compared by Student's *t*-test, differences were considered statistically significant when p values were less than 0.05.

RESULTS

Phospholipid content of rat renal papilla, medulla and cortex. Different amounts of endogenous PL were found in the three zones of the kidney (Table 1). The total phospholipid content (expressed as nmol/mg tissue wet weight) was 5.6, 15.8 and 20.7 in papilla, medulla and cortex, respectively. No changes in these values were observed after a 90 min incubation at 37°C. The profiles of the various phospholipids were different depending on the zone studied. In the papilla (P), PtdCho and PtdEtn represented 46.4% and 26.5%, respectively, of total PL. The percentages of PtdIns and PtdSer were both 8.8, while sphingomyelin constituted 9.1%. In the medulla (M), the percentages of the different PL classes were: PtdCho, 37.8; PtdEtn, 39.4; PtdSer, 7.4; PtdIns, 3.9; and Cer*P*Cho, 12.2. In the cortex, PtdCho represented 41.4%; PtdEtn, 32.5%; PtdSer, 5.8%; PtdIns, 3.6%; and Cer*P*Cho, 16.5%.

Incorporation of [2-^{14}C]glycerol in papillary, medullary and cortical phospholipids. The incorporation of [2-^{14}C]-glycerol ([2-^{14}C]Gro) into the kidney phospholipids after 90 min of incubation is shown in Figure 1. The insertions in the Figures show specific activities as cpm/nmol PL. In each zone studied, the highest incorporation (cpm/mg tissue wet weight) was found in PtdCho, followed by PtdIns, and the lowest value was observed in PtdEtn. No radioactivity was associated with PtdSer.

When the results are expressed as specific activity (cpm/nmol PL), PtdIns showed the highest incorporation. The ratio of PtdIns/PtdCho increased gradually from papilla to cortex (1.6, 4.5 and 9.3 for P, M and C, respectively).

The radioactivity incorporated into PtdEtn was lower than that incorporated into PtdIns in the three zones and, when the results are expressed as specific activities, the differences are exacerbated. No radioactivity had been found in PtdSer, indicating that this phospholipid must

TABLE 1

Phospholipid Content of Rat Renal Papillary, Medullary and Cortical Slices[a]

PL	Papilla	Medulla	Cortex
PtdCho	2.60 ± 0.32	5.96 ± 0.67[b]	8.58 ± 1.00[b]
PtdEtn	1.49 ± 0.21	6.24 ± 0.94[b]	6.73 ± 0.95[b]
PtdSer	0.49 ± 0.10	1.18 ± 0.09[b]	1.19 ± 0.10[b]
PtdIns	0.49 ± 0.09	0.62 ± 0.11	0.75 ± 0.09[b]
Cer*P*Cho	0.50 ± 0.07	1.78 ± 0.19[b]	3.42 ± 0.45[b]
Phospholipid content after 90 min incubation (37°C)			
PtdCho	2.63 ± 0.29	5.66 ± 0.71[b]	8.43 ± 1.10[b]
PtdEtn	1.39 ± 0.26	6.11 ± 1.00[b]	6.75 ± 0.98[b]
PtdSer	0.51 ± 0.09	1.15 ± 0.10[b]	1.21 ± 0.11[b]
PtdIns	0.50 ± 0.10	0.59 ± 0.12	0.72 ± 0.10[b]
Cer*P*Cho	0.48 ± 0.08	1.79 ± 0.18[b]	3.40 ± 0.50[b]

[a]Tissue slices were homogenized and lipids were extracted and separated by thin-layer chromatography. Results are means ± SE; for each value n = 5. Data are expressed in nmol/mg of tissue wet weight.

[b]Significantly different ($p<0.05$) from that of papilla.

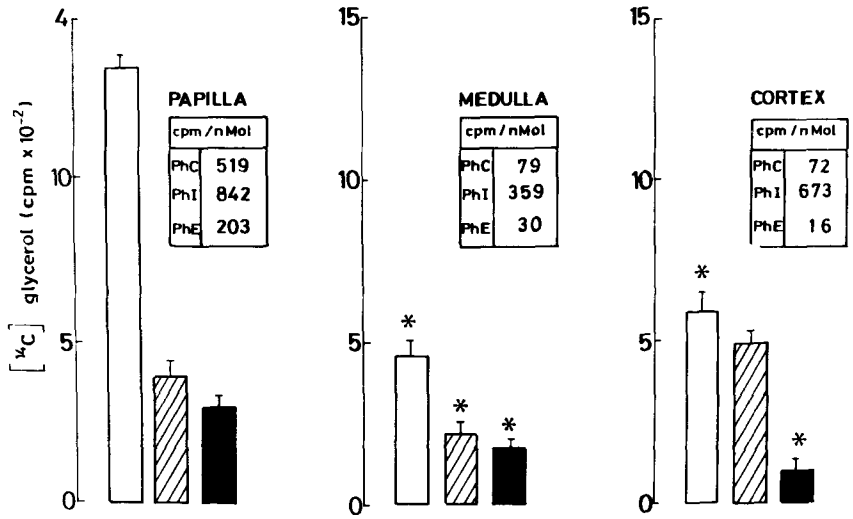

FIG. 1. Incorporation of [2-^{14}C]glycerol into phospholipids of rat renal papilla, medulla and cortex. Tissue slices were incubated for 90 min at 37°C in the presence of 0.12 μCi of [2-^{14}C]Gro. Phospholipids were extracted and separated as described in Materials and Methods. Each bar represents the means ± SE of five experiments (cpm/mg tissue wet weight). PtdCho, white bar; PtdIns, hatched bar; and PtdEtn, black bar. *, Significantly different ($p<0.05$) from the same phospholipid in papilla.

come from an exchange process, possibly derived from plasma phospholipids.

Incorporation of [^{14}C]fatty acids into rat renal papillary, medullary and cortical phospholipids. [1-^{14}C]-Palmitic acid ([1-^{14}C]16:0) can be incorporated into PL by *de novo* synthesis or by the deacylation-reacylation cycle. This saturated fatty acid was incorporated into position 1 of glycerol. By contrast, arachidonic acid (AA) is an essential polyunsaturated fatty acid that is incorporated into PL only during phospholipid remodelling that occurs *via* deacylation-reacylation.

Figure 2 shows the incorporation of [1-^{14}C]16:0 at 60 min of incubation. Most of the radioactivity was incorporated into PtdCho in the three zones. In papilla and medulla, almost ten times more radioactivity was found in PtdCho than in PtdEtn. In cortex, the amount of radioactivity in PtdEtn was 1/5 that of PtdCho.

[1-^{14}C]16:0 Acyl incorporation into PtdIns was very low and showed the lowest levels of radioactivity. In terms of specific activity, PtdCho seemed to be the most active phospholipid, followed by PtdIns and then by PtdEtn. No radioactivity was associated with PtdSer.

The pattern of [1-^{14}C]AA incorporation (Fig. 3) was different from that of the saturated fatty acid.

Although more radioactivity was found in phosphatidylcholine than in PtdIns and PtdEtn, when the results are expressed as specific activity the value obtained for PtdCho was two (papilla) to three times (medulla and cortex) lower than that for PtdIns. [1-^{14}C]AA radioactivity associated with phosphatidylethanolamine was similar to that obtained in phosphatidylinositol in medulla and cortex, but the specific activities were ten (medulla) or more (cortex) times lower. In papilla, the incorporation of radioactive arachidonic acid into phosphatidylethanolamine was 1/2 that of phosphatidylinositol.

DISCUSSION

Table 1 shows that papillary, medullary and cortical phospholipids differ both in concentration and in distribution profiles. Papilla is the tissue that possesses the highest phosphatidylinositol and the lowest sphingomyelin levels. These two Pl are particularly important because of their functional implications. Thus, it has been reported that Cer*P*Cho inhibits both PtdCho breakdown brought about by the deacylating phospholipase A_2 (13) and the non-stimulated activity of phosphatidylinositol phosphodiesterase (14). For these reasons, it has been proposed that the enhanced content of Cer*P*Cho in the membrane bilayer tones down the action of cellular phospholipases (13). Phosphatidylinositol has been proposed as a link between hormone receptors and cellular response (15). In the kidney, PtdIns breakdown is brought about by a specific phosphodiesterase which precedes the arachidonic acid mobilization produced by bradykinin stimulation (16).

The relative amount of Cer*P*Cho and PtdIns in the membrane bilayer may regulate some aspects of its phospholipid metabolism as is discussed below.

The two main pathways in kidney phospholipid metabolism are *de novo* synthesis (17) and the deacylation-reacylation cycle (18). [2-^{14}C]Gro enters the phospholipid molecule only by *de novo* synthesis (19). Therefore, our results (Fig. 1) suggest that most *de novo* synthesis of active phospholipids occurs in the papilla. However, the activity of the glycerol kinase (the first enzyme in glycerol metabolism) is over ten times lower in papilla than in medulla and cortex (20,21). One possible explanation for this is that cortex and medulla are very efficient in using the glycerol phosphate formed to produce glucose and lactate, respectively. By contrast, glycerol does not serve as precursor of glucose or lactate in papilla because the

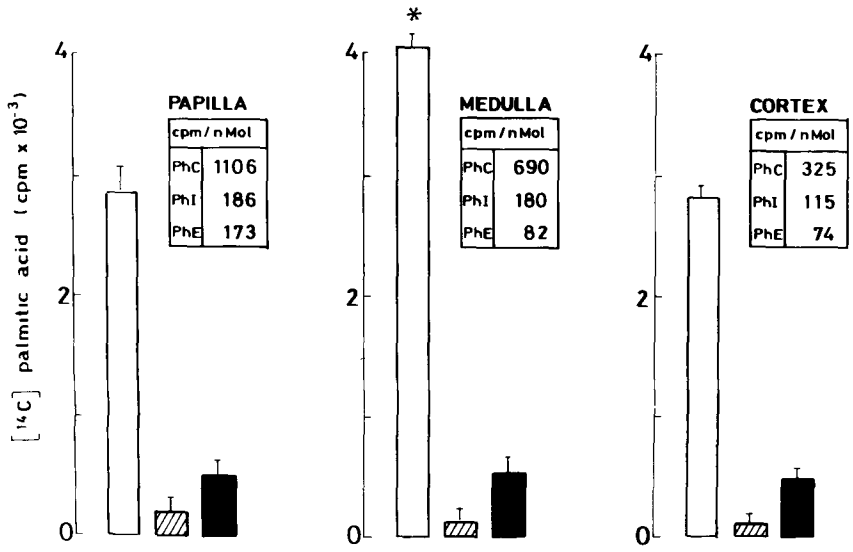

FIG. 2. Incorporation of [1-^{14}C]palmitic acid into phospholipids of rat renal papilla, medulla and cortex. Tissue slices were incubated for 60 min at 37°C in the presence of 0.12 μCi of [2-^{14}C]16:0. Phospholipids were extracted and separated as described in Materials and Methods. Each bar represents the means ± SE of five experiments (cpm/mg tissue wet weight). PtdCho, white bar; PtdIns, hatched bar; and PtdEtn, black bar. *, Significantly different ($p<0.05$) from the same phospholipid in papilla.

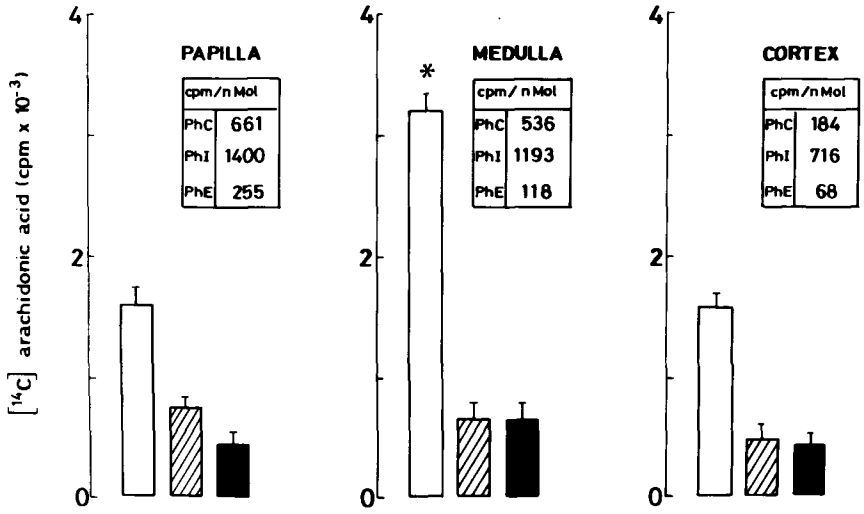

FIG. 3. Incorporation of [1-^{14}C]arachidonic acid into phospholipids of rat renal papilla, medulla and cortex. Tissue slices were incubated for 60 min at 37°C in the presence of 0.12 μCi of [1-^{14}C]AA. Phospholipids were extracted and separated as described in Materials and Methods. Each bar represents the means ± SE of five experiments (cpm/mg tissue wet weight). PtdCho, white bar; PtdIns, hatched bar; and PtdEtn, black bar. *, Significantly different ($p<0.05$) from the same phospholipid in papilla.

gluconeogenic enzymes and the pyruvate kinase necessary to form glucose and lactate, respectively, are not present (21). In consequence, glycerol phosphate may be more available for phospholipid biosynthesis. Results from our experiments with [1-^{14}C]palmitic and [1-^{14}C]arachidonic acid (Figs. 2 and 3) suggest that the incorporation profile for the two fatty acids is different. While phosphatidylcholine is the most active phospholipid for palmitic acid incorporation, phosphatidylinositol shows the highest specific activity for arachidonic acid in the three zones. The highest value described in the insertions in Figure 3 for all phospholipids correspond to papilla. Incorporation of arachidonic acid occurs by deacylation-reacylation. The first step in the cycle is the release of the fatty acid in position two (deacylation) by an endogenous phospholipase A_2. The efficient incorporation of arachidonic acid may, in part, be due to the low level of papillary sphingomyelin, which enhances lipase cleavage and, as a consequence, provides more substrate for the incorporation of fatty acid (reacylation).

The incubation time used in experiments with radioactive precursors corresponds to the steady-state period of the most metabolically active pools and does not include the total phospholipid pool. Still these results are relevant because this active pool may be easy to modify and may be the substrate for the changes induced by endogenous hormones and neurotransmitters that regulate cellular function.

The results clearly indicate that the zones of the kidney differ in phospholipid properties. The papilla, where the terminal segment of the nephron is located, is involved in the final adjustments of urine volume and composition. It is the zone where less endogenous phospholipid is present, but it is also where its turnover is more active. Phosphatidylinositol is the most active phospholipid in the three zones studied except for its ability to incorporate palmitic acid.

Evidence has been presented that many of the cells' physiological activities are strongly influenced by membrane phospholipid composition. The observation that induction of changes in lipid composition can impair membrane function is evidence of this interrelationship (22).

In conclusion, we found that non-stimulated cells with different phospholipid compositions also differ in some aspects of phospholipid metabolism. We suggest that such differences in lipid profile are responsible, at least in part, for the metabolic dissimilarities.

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Lipids of Selected Molds Grown for Production of n-3 and n-6 Polyunsaturated Fatty Acids

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The lipid classes and component fatty acids of seven fungi were examined. Three marine fungi, *Thraustochytrium aureum, Thraustochytrium roseum* and *Schizochytrium aggregatum* (grown at 30, 25 and 25°C, respectively), produced less than 10% lipid but contained docosahexaenoic acid (DHA) up to 30% and eicosapentaenoic acid (EPA) up to 11% of the total fatty acids. *Mortierella alpina-peyron* produced 38% oil containing solely n-6 polyunsaturated fatty acids (PUFA) with arachidonic acid (AA) at 11% of the total fatty acids. *Conidiobolus nanodes* and *Entomorphthora exitalis* produced 25% oil and contained both n-3 and n-6 PUFA, with AA at 16% and 18%, respectively. *Saprolegnia parasitica* produced 10% oil and contained AA and EPA, respectively, at 19% and 18%. The triacylglycerol fraction always represented the major component at between 44% and 68% of the total lipid. Each fungus, except *T. aureum*, had the greatest degree of fatty acid unsaturation in the phospholipid fraction. The triacylglycerol fraction of *T. aureum* was the most unsaturated with DHA representing 29% (w/w) of all fatty acids present. The presence of the enzyme ATP:citrate lyase correlated with the ability of molds to accumulate more than 10% (w/w) lipid when the fungi were grown in nitrogen-limiting media. In those molds that failed to accumulate more than 10% lipid, the enzyme was absent.
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In recent years, nutritional studies have shown the potential benefits of including long-chain polyunsaturated fatty acids (PUFA) in the diet. Evening primrose oil, which contains about 8% (w/w) gamma-linolenic acid (18:3n-6) has long been recommended for the treatment of a wide variety of ailments (1). Other recent work has concentrated on PUFA of the n-3 series, mainly eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) which may be beneficial in the prevention of coronary heart disease (2).

Certain fungi, referred to as oleaginous species, have the ability to accumulate up to 85% (w/w) lipid as a storage compound of the biomass (3). This accumulation is triggered by exhaustion of a growth nutrient, other than carbon, thus preventing cell proliferation but still allowing conversion of substrate into lipid (3). Such oleaginous fungi, which produce PUFA of both n-6 and n-3 species, could provide an economically viable source of PUFA providing that most of the PUFA occur in the triacylglycerol fraction of lipids which would then be extractable as a plant-like oil rather than a complex array of lipid classes.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; GLC, gas-liquid chromatography; PUFA, polyunsaturated fatty acids; TAG, triacylglycerols; TLC, thin-layer chromatography; UV, ultraviolet.

The organisms used in this study were carefully selected as being potentially useful for the production of PUFA, though it was not known whether these organisms were oleaginous. The marine fungi, *Thraustochytrium aureum, Thraustochytrium roseum* and *Schizochytrium aggregatum*, were examined because of their low natural growth temperature which should lead to a high PUFA content of the cellular lipids (4) as had been reported (5). No report has appeared to indicate how much lipid can be maximally accumulated by these species. *Saprolegnia parasitica* was examined as it has been reported to produce up to 10% (w/w) of total fatty acids as arachidonic acid and 20% (w/w) as eicosapentaenoic acid (6) in its extracted lipids.

Although a number of species of *Mortierella* have been recently examined by Japanese groups for PUFA formation (7–9), these species have all belonged to the sub-genus, *Mortierella mortierella*, with no attention being given to any representative of the other sub-genus, *Mortierella micromucor*. Accordingly, we have included in our study a representative of the latter group, *Mortierella alpina-peyron*, to see whether it is similar to members of the other sub-genus in the potential formation of arachidonic and eicosapentaenoic acids.

Fatty acids of the *Conidiobolus* and *Entomorphthora* were originally examined by Tyrrell (10–12) with the view to using this data as an aid for fungal classification. Both classes of fungi were reported to contain PUFA and were hence included in this study but their potential as oleaginous species has not before been fully explored.

MATERIALS AND METHODS

Micro-organisms and growth. The fungal strains examined were *Conidiobolus nanodes* (IMI 92299), *Thraustochytrium aureum* (ATCC 34304), *T. roseum* (ATCC 28210), *Schizochytrium aggregatum* (ATCC 28209), *Saprolegnia parasitica* (ATCC 22284), *Entomorphthora exitalis* (NRRL 3742) and *Mortierella alpina-peyron* (CBS 696.70). Fungi were cultivated using vegetative inocula in vortex-aerated, 1-L bottles with 800 mL semi-defined medium which contained (g/L) glucose, 30; ammonium tartrate, 3.3; KH_2PO_4, 7.0; Na_2HPO_4, 2; $MgSO_4 \cdot 7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2 \cdot 2H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.008; $ZnSO_4 \cdot 7H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.0001; $Co(NO_3)_2 \cdot 6H_2O$, 0.0001; $MnSO_4 \cdot 5H_2O$, 0.001; with pH adjusted to 5.5. The marine fungi *T. aureum, T. roseum* and *Schizo. aggregatum* were grown in the same medium supplemented with 27 g NaCl/L and $MgSO_4 \cdot 7H_2O$ increased to 7 g/L. *T. aureum, C. nanodes,* and *E. exitalis* were grown at 30°C; *M. alpina-peyron* was grown at 28°C; *T. roseum* and *Schizo. aggregatum* at 25°C, and *Sapro. parasitica* at 24°C.

Lipid content determination. The lipid content of the fungi was determined after 72-hr growth. The mycelial fungi and non-mycelial fungi were harvested by vacuum filtration and by centrifugation (10,000 × *g* for 10 min at 4°C). Harvested fungi were washed once with distilled

water (100 mL), frozen to −20°C and then freeze-dried for 18 hr. The mycelia were ground into a fine powder and lipids were extracted into 100 mL chloroform/methanol (2:1, v/v) for 15 hr at room temperature (13). The lipid extract was washed with distilled water (3 × 20 mL) and dried over anhydrous $MgSO_4$; the solvent was removed by evaporation.

Fractionation of extracted lipid. A known weight of extracted lipid (approx. 100 mg) was dissolved in chloroform (1 mL) and fractionated by using a column (25 × 100 mm) of silicic acid (activated by heating at 110°C overnight) (14). Successive applications of 1,1,1-trichloroethane (100 mL), acetone (100 mL) and methanol (50 mL) produced fractions containing neutral lipids (triacylglycerols), sphingolipids plus glycolipids, and polar lipids (phospholipids), respectively. The weight of each fraction was determined. Recoveries were usually between 85 and 90% (w/w).

Hydrogenation of lipid extract. Lipid (100 mg) from *T. aureum* was dissolved in chloroform/methanol (1:1, v/v), mixed with 0.25 g 5% (w/w) Pd/charcoal catalyst (BDH, Poole, Dorset, United Kingdom) vigorously stirred in an atmosphere of hydrogen at atmospheric pressure for 2 hr. The catalyst was then removed by filtration through Celite filter aid (BDH). Excess solvent was removed by rotary evaporation, and the sample was treated as before.

Thin-layer chromatography of lipid fractions eluted from sililic acid column. Lipid fractions were separated by thin-layer chromatography (TLC) using Silica Gel G plates (0.25 mm thick) developed with petroleum ether (b.p. 60–80°C)/diethyl ether/acetic acid (85:15:1, v/v/v). Plates were exposed to iodine vapor to reveal the neutral lipids, or were sprayed with 2′,7′-dichlorofluorescein (0.2% w/v in ethanol) and viewed under ultraviolet (UV) light for the polar lipid fraction, or were sprayed with α-naphthol or Dragendorff reagent for the sphingolipid plus glycolipid fraction. Identifications were made by reference to authentic standards.

Detection of glycolipids using α-naphthol (15). Following development and drying of the chromatogram, the TLC plate was sprayed with α-naphthol solution (0.5% w/v in methanol/water, 1:1 v/v). After drying, the plate was sprayed lightly with sulfuric acid (95% v/v) and heated at 120°C for 5 min after which time glycolipids turned blue.

Detection of choline-containing lipids using the Dragendorff reagent (16). Four parts of solution 1 (2.7% bismuth nitrate in 20%, v/v, acetic acid) were mixed with 1 part of solution 2 (40% w/v potassium iodide), and the resulting solution was diluted with 14 parts of water. The developed and dried chromatogram was sprayed with the Dragendorff reagent and choline containing lipids stained orange/red.

Further fractionation of phospholipid fraction obtained from E. exitalis *and* C. nanodes *(14).* Phospholipids were analyzed by TLC on Silica Gel G plates (0.25 mm thick) developed with chloroform/methanol/acetic acid/water (65:43:3:1, by vol), visualized with 2′7′-dichlorofluorescein, and viewed under UV light.

Fatty acid analysis. Fractionated lipid extracts were transesterified with sodium methoxide (17), and the fatty acid methyl esters (FAME) were analyzed by gas-liquid chromatography using a 2 m × 4 mm column packed with diethyleneglycol succinate (10% w/w on Celite); nitrogen was used as the carrier gas (35 mL/min). FAME were identified by reference to authentic standards and a graph of chain length against retention time (for each degree of unsaturation).

Preparation of cell-free extracts. Cultures were grown for 72 hr and harvested. The mycelia were resuspended in extraction buffer which contained 30 mM Tris/HCl pH 7.8, 2 mM dithiothreitol, 2 mM benzamidine/HCl, 1 mM EDTA, and 20% (v/v) glycerol. Cells were disrupted by two passages through a French pressure cell. The supernatant solution was centrifuged at 48000 × *g* for 20 min and then filtered through Whatman no. 1 paper to remove lipid material. The filtrate was re-centrifuged (100,000 × *g* for 1 hr) and again refiltered to remove final traces of lipid. This filtrate was termed "the cell-free extract." The concentration of the protein in the cell-free extract was determined by the method of Bradford (18).

ATP:citrate lyase (E.C. 4.1.3.8) assay (19). The following were added to a 1 mL cuvette (containing up to 0.42 mL cell-free extract) to produce the final concentrations indicated: 10 mM Tris/HCl pH 8.2; 10 mM $MgCl_2$; 20 mM potassium citrate; 0.3 mg CoA/mL; 10 mM dithiothreitol; 0.125 mg NADH/mL; 500 units malic dehydrogenase/mL. The reaction was initiated by adding 10 mM ATP. The mixture was incubated at 20°C and the reaction was followed at 340 nm.

RESULTS AND DISCUSSION

Growth and lipid accumulation of fungi. Two of the marine fungi, *T. roseum* and *Schizo. aggregatum,* grew only sparingly in the glucose/ammonium tartrate medium. The cells exhibited a non-mycelial, yeast-like morphology. Their respective biomasses were 1.2 and 1.4 g dry weight/L and they produced very little lipid (0.5% and 1.7% w/w, respectively). Attempts to improve these results by including Bacto-peptone, malt extract or glutamate, or by varying the salinity of the growth medium, did not increase the amounts of lipid formed. The reason for the very low amount of lipid accumulated by the fungi *T. roseum* and *Schizo. aggregatum* is not known but may well be connected with the poor growth of the organisms.

By contrast, the third marine fungus, *T. aureum,* grew (to 4 g dry weight/L) readily in the same basal medium (see Materials and Methods) used for the other two marine organisms. The fungus grew as a pink/purple non-mycelial, yeast-like culture but never exceeded a content of 10% (w/w) lipid.

All other fungi grew readily as dispersed mycelial cultures. The growth yields and lipid contents are given in Table 1.

The efficiency of lipid extraction was tested by refluxing the extracted mycelial residues of *C. nanodes* and *E. exitalis* with chloroform/methanol (2:1, v/v) for 1 hr. The results showed that the initial chloroform/methanol treatment extracted 94% (w/w) of total cellular lipids.

Fractionation and analysis of fungal lipid extracts. The extracted lipids were fractionated as detailed in Materials and Methods. Analysis of each fraction by TLC showed that the neutral lipid fraction (eluted with 1,1,1-trichloroethane) contained predominantly triacylglycerols and small amounts (less than 10%) of diacylglycerols and monoacylglycerols. Triacylglycerols (TAG) represented 68 to 44% (w/w) of total extracted lipid from both oleaginous and non-oleaginous fungi. These results are also summarized in Table 1.

TABLE 1

Growth and Lipid Accumulation by Fungi[a]

Fungus	Biomass (g dry weight/L)	Lipid content (%, w/w)	Percentage of each lipid fraction present (%,w/w)		
			N	S + G	P
T. aureum	4.0	10.0	64.0	15.0	21.0
T. roseum	1.2	0.5	44.7	27.1	28.2
Schizo. aggregatum	1.4	1.7	48.0	27.1	22.9
Sapro. parasitica	8.1	10.1	53.2	14.1	32.9
C. nanodes	9.8	25.7	68.2	21.5	10.3
E. exitalis	11.6	24.6	57.2	20.5	22.3
M. alpina-peyron	3.2	37.7	52.6	18.4	28.9

[a]Abbreviations used: N, neutral lipids; S + G, sphingolipids plus glycolipids; P, phospholipids.

The polar lipid fractions (eluted with methanol) from each organism contained only phospholipids. More detailed analysis of the fraction from the lipid extract of *C. nanodes* showed phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine to be present in approximately equal proportions. Phosphatidylcholine accounted for approx. 80% of the total phospholipids in *E. exitalis*.

TLC of the sphingolipid plus glycolipid fraction obtained from *E. exitalis* and *C. nanodes* revealed, respectively, the presence of four and five components which were not identified further beyond establishing that two or three were sphingolipids and two contained a sugar moiety.

Fatty acid analysis of lipid extracts. The seven fungi examined produced an exceptionally large number of fatty acids, many of which contained two or more double bonds (*i.e.*, are classified as PUFA). These results are summarized in Tables 2–4. However, although a large number of fatty acids were found in every lipid fraction, one PUFA (two in the case of *Sapro. parasitica*) predominated in each fungal lipid.

C. nanodes and *E. exitalis* produced both n-3 and n-6 PUFA but the n-6 PUFA were in greater abundance. The

TABLE 2

Fatty Acid Profile of the Neutral Lipid Fraction Extracted from Fungi[a]

Fatty acid[a]	*T. aureum*	*T. roseum*	*Schizo. aggregatum*	*Sapro. parasitica*	*C. nanodes*	*E. exitalis*	*M. alpina-peyron*
Saturated							
14:0	tr	1.6	3.8	15.5	1.1	9.3	9.6
16:0	9.7	18.1	16.5	21.1	22.7	17.2	14.6
17:0	tr	0.7	tr	0.6	—	0.9	1.9
18:0	2.4	7.8	5.8	11.4	15.0	2.8	7.1
20:0	tr	—	tr	1.7	—	0.2	8.4
Monosaturated							
14:1	—	1.1	1.1	2.3	0.9	0.2	0.2
16:1	0.3	2.3	5.7	4.7	1.6	12.7	4.3
18:1	26.1	31.4	41.2	20.5	24.8	41.0	29.8
20:1	tr	—	—	0.4	13.0	—	1.4
22:1	—	—	—	—	8.1	—	—
PUFA							
16:2	tr	4.1	1.5	0.6	0.4	0.8	0.5
182	7.0	9.3	15.0	9.7	1.2	4.8	9.4
18:3n-3	1.1	3.1	3.2	—	0.4	0.2	—
18:3n-6	—	1.3	tr	1.4	3.8	0.9	1.3
20:2	tr	0.8	0.7	—	0.5	0.2	—
20:3n-6	tr	1.2	—	1.8	0.4	0.4	6.1
20:4n-6	—	3.8	tr	3.8	3.9	3.8	5.4
20:5n-3	8.8	4.6	1.3	3.9	tr	0.9	—
22:2	—	—	—	—	—	—	—
22:4	—	—	—	0.6	—	—	—
22:5n-3	7.2	0.6	tr	—	0.1	1.2	—
22:6n-3	29.9	5.7	4.2	—	2.3	1.2	—
24:2	5.6	4.0	—	—	—	—	—
26:2	2.5	—	—	—	—	—	—

[a]Expressed as percentages (w/w) of total fatty acids present; tr, less than 0.11 present.

TABLE 3

Fatty Acid Profile of the Sphingolipid and Glycolipid Fraction Extracted from Fungi

Fatty acid	*T. aureum*	*T. roseum*	*Schizo. aggregatum*	*Sapro. parasitica*	*C. nanodes*	*E. exitalis*	*M. alpina-peyron*
Saturated							
14:0	0.3	14.7	2.1	11.1	1.9	9.9	7.1
16:0	11.6	24.6	17.0	21.3	22.6	15.1	13.5
17:0	—	—	—	tr	—	0.6	—
18:0	16.2	3.5	6.3	8.2	12.4	4.9	9.7
20:0	0.3	1.1	—	0.9	—	3.5	—
Monosaturated							
14:i	—	1.4	0.8	0.6	0.1	1.2	tr
16:1	0.1	5.5	12.3	3.9	3.0	4.3	4.7
18:1	33.7	29.0	24.6	23.3	28.2	31.2	48.8
20:1	—	0.7	—	0.7	10.7	—	—
22:1	—	0.1	—	—	5.3	—	—
PUFA							
16:2	tr	2.0	—	0.3	0.4	0.2	tr
18:2	16.4	10.6	12.4	12.3	1.2	9.7	15.1
18:3n-3	1.0	tr	0.8	—	0.4	3.5	—
18:3n-6	—	tr	3.0	1.0	2.5	2.9	tr
20:2	—	—	2.9	—	tr	0.6	—
20:3n-6	—	—	2.9	1.3	2.0	0.6	—
20:4n-6	—	tr	3.0	5.7	8.4	4.8	1.1
20:5n-3	10.8	3.4	6.7	8.8	0.3	4.2	—
22:2	—	—	0.6	—	—	—	—
22:4	—	—	—	—	—	—	—
22:5n-3	—	1.1	1.5	—	0.3	tr	—
22:6n-3	9.3	3.8	4.1	—	0.8	3.0	—
24:2	tr	tr	0.5	—	—	—	—
26:2	0.1	—	—	—	—	—	—

TABLE 4

Fatty Acid Profile of Phospholipid Fractions Extracted from Fungi

Fatty acid	*T. aureum*	*T. roseum*	*Schizo. aggregatum*	*Sapro. parasitica*	*C. nanodes*	*E. exitalis*	*M. alpina-peyron*
Saturated							
14:0	0.6	12.2	4.8	5.3	0.2	9.6	4.4
16:0	9.1	25.4	10.1	14.8	25.8	24.0	14.2
17:0	tr	1.9	—	0.1	—	1.7	—
18:0	4.4	2.2	8.0	2.6	0.7	1.8	1.9
20:0	3.7	—	—	1.7	—	tr	0.2
Monosaturated							
14:1	0.4	—	tr	0.6	1.1	1.4	0.1
16:1	0.4	1.6	3.6	2.4	1.1	11.2	6.2
18:1	30.0	20.2	19.8	18.7	25.8	18.0	24.2
20:1	tr	—	—	0.1	4.8	—	0.5
22:1	—	—	—	—	4.1	—	—
PUFA							
16:2	tr	6.8	1.8	0.2	tr	0.7	0.5
18:2	18.7	10.9	11.9	11.9	3.0	9.9	17.1
18:3n-3	2.5	2.8	1.1	—	0.9	tr	—
18:3n-6	tr	1.9	tr	1.9	1.8	3.3	4.6
20:2	—	0.1	—	—	0.4	0.2	2.6
20:3n-6	—	0.1	1.0	2.3	1.2	0.7	3.3
20:4n-6	tr	0.3	1.5	19.0	15.2	17.5	10.7
20:5n-3	9.9	0.1	15.9	18.0	1.7	tr	—
22:2	—	—	—	—	—	—	—
22:4	—	—	—	0.2	—	—	—
22:5n-3	2.9	1.3	7.7	—	2.3	—	—
22:6n-3	16.8	8.5	12.4	—	1.6	tr	—
24:2	1.3	3.1	0.7	—	—	—	—
26:2	0.1	—	—	—	—	—	—

predominating n-6 PUFA produced was arachidonic acid (20:4n-6), which in the phospholipids of these fungi represented 15% and 18%, respectively, of the fatty acids in this lipid class.

The marine fungus *T. aureum* produced only n-3 PUFA and docosahexanenoic acid (DHA) represented 30% of total fatty acids in TAG.

M. alpina-peyron produced n-6 PUFA only. Similar to the lipid from *C. nanodes* and *E. exitalis,* this lipid contained arachidonic acid (AA) as the principal PUFA; AA represented 11% (w/w) of the fatty acyl groups in the phospholipids.

The two classes of PUFA discussed above are derived from the two isomers of linolenic acid. The alpha isomer (18:3n-3) is the fatty acid from which the n-3 PUFA are directly synthesized. The gamma isomer (18:3n-6) is similarly the parent fatty acid of the n-6 PUFA series. The relative proportion of the two classes of PUFA present in each fungus is reflected in the ratios of the isomers of linolenic acid.

The exception to the above pattern of PUFA is *Saprolegnia parasitica* which contained only one n-3 PUFA eicosapentaenoic acid, though many n-6 PUFA were present as well. These included gamma-linolenic acid, dihomogamma-linolenic acid and arachidonic acid. This finding of only one n-3 PUFA amidst many n-6 PUFA supports the proposal of Gellerman and Schlenk (6) who demonstrated the conversion of AA into eicosapentaenoic acid (EPA) *via* a Δ17 desaturase in *Saprolegnia parasitica.* As yet, this is the only known point of conversion between the two classes of a PUFA, and occurred in *M. alpina* when grown at low temperatures (7). Interestingly *Sapro. parasitica* was the only fungus examined here that metabolized arachidonic acid further by elongation to 22:4n-6.

In all the fungi examined, except *T. aureum,* the phospholipid fraction contained the greatest proportion of PUFA. This was true for both classes of PUFA. Phospholipids are highly specialized membrane components which regulate membrane fluidity in response to high salinities, temperature variations, etc. In turn, this allows the range of complex membrane functions to continue. The high degree of unsaturation of the fatty acyl groups in the phospholipids may be a structural necessity of their function as membrane components. The desaturases in micro-organisms and all cells so far examined have been membrane-bound (20–22), and strong evidence suggests that fatty acids are desaturated when attached to phospholipids rather than as the thiol ester of CoA (20). Once the necessary desaturation has occurred, acyl transferase reactions then facilitate distribution of the newly synthesized PUFA to other cellular lipids.

A high concentration of PUFA within the polar lipid fraction was also evident in the marine fungus *T. aureum.* DHA made up 17% (w/w) of total fatty acids and 30% of TAG. The high concentration of DHA in TAG of this organism may make it a potentially useful source for DHA production. However changes in growth medium and growth conditions have, so far, failed to increase either the amount of total lipid produced or the content of DHA (Kendrick, A., unpublished work).

The long-chain PUFA, 24:2 and 26:2, found in the marine fungi were tentatively identified from their initial retention times in gas-liquid chromatography (GLC) and also those of the saturated fatty acids (24:0 and 26:0) obtained by hydrogenation of the lipids extracted from *T. aureum.* (The number of double bonds in the original fatty acids was calculated from a graph of chain length against log retention time.) No work as to the location of the double bonds within these molecules has been undertaken. The metabolic role of 24:2 and 26:2 is unclear though the occurrence of 24:2 in all three of the marine fungi examined here might suggest a role in halotolerance. No other C_{24} or C_{26} fatty acids were present in the marine fungi.

TABLE 5

Correlation of Lipid Accumulation in Molds with ATP:Citrate Lyase Activity

Fungus	Lipid content % (w/w)	ATP:citrate lyase activity (nmol/min/mg protein)
T. aureum	10.0 ± 1.9	0
T. roseum	0.5 ± 0.1	0
Schizo. aggregatum	1.7 ± 0.2	0
Sapro. parasitica	10.1 ± 0.8	0
C. nanodes	25.7 ± 0.5	19
E. exitalis	24.6 ± 0.5	11
M. alpina-peyron	37.7 ± 2.3	18

ATP:citrate lyase activity of fungi. Activity of the enzyme ATP:citrate lyase was detected in cell-free extracts prepared from the fungi *E. exitalis, C. nanodes* and *M. alpina-peyron* (Table 5). These three fungi can be termed oleaginous because they accumulated over 20% lipid (25%, 26% and 38% w/w lipid, respectively). No activity of the enzyme was found in any of the marine fungi or *Sapro. parasitica* (Table 5), a non-oleaginous species.

ATP:citrate lyase is associated with lipid accumulation in oleaginous yeasts (23). The present results concur with the hypothesis that when this activity is absent, lipid accumulation is likely to be low. The maximum lipid content attained by a fungus lacking this enzyme was 10% (w/w). Thus, fungi with ATP:citrate lyase activity should, theoretically, be metabolically capable of accumulating substantial amounts of lipid.

M. alpina-peyron, the organism which accumulated the greatest amount of cellular lipid (38% w/w), produced the smallest proportion of PUFA. Conversely, organisms which produced the largest amount of C_{20} and C_{22} PUFA, *Sapro. parasitica* and *T. aureum,* respectively, only accumulated low amounts of lipid and, by the absence of the enzyme ATP:citrate lyase, would appear constitutionally unable to achieve lipid accumulation beyond these amounts.

Any organism chosen for the commercial production of a given PUFA would ideally be both oleaginous and produce a triacylglycerol oil rich in the desired fatty acid. None of the fungi examined, however, fit these criteria. Prospects of increasing the oil contents (and thus the TAG content) of a mold must be confined to those with a propensity for oil accumulation as indicated by the presence of ATP:citrate lyase, the key enzyme for lipid accumulation (23).

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Note added in proof. After submission of this paper, L5986, two related publications (Bajpai, P., Bajpai, P.K. and Ward, O.P. (1991) *Appl. Microbiol. Biotechnol. 35,* 706-710; Bajpai, P.K., Bajpai, P., and Ward, O.P. (1991) *J. Am. Oil Chem. Soc. 68,* 509-514) have appeared reporting the production of docosahexaenoic acid (DHA) by *Thraustochytrium aureum* ATCC 34304 grown under different regimens. Maximum lipid contents of 20% (w/w) of biomass were recorded, with DHA in total lipid amounting to up to 50% of total fatty acids. Although these data represent slight improvements on the results reported here, the fungus continued to grow slowly reaching only 5 g of biomass/L after 144 hr. Thus our conclusions (see last paragraph) still hold good.

Linoleoyl-Enriched Triacylglycerol Species Increase in Maternal Liver During Late Pregnancy in the Rat

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In view of the previously reported changes in the fatty acid composition of maternal liver triacylglycerols in late pregnancy, changes in the composition of maternal liver triacylglycerol species were assessed in rats fed a semi-purified diet during pregnancy. Between day 18 and day 21 of pregnancy, total maternal liver triacylglycerols increased by 50%. Triacylglycerol species with a total acyl carbon number (C) of 50 or 60 (C50, C60) remained unchanged while C48 and C52–C58 were relatively increased. The individual triacylglycerol species containing one, two or three linoleoyl moieties were incompletely recovered using a polar high temperature gas-liquid chromatography (GLC) column. Nevertheless, at day 21 compared to day 18, the linoleoyl-containing species were relatively increased by 62–463%, while tripalmitin was decreased by 38%. Our data suggest that despite an adequate intake of linoleic acid (25 g/kg in the diet), maternal hepatic triacylglycerol content of linoleic acid decreased during mid-pregnancy but increased significantly toward term possibly in preparation for the transfer of linoleic acid to the neonate during lactation.
Lipids 27, 21-24 (1992).

Pregnancy involves significant metabolic adaptations by the mother to the developing conceptus. These metabolic adaptations include transfer from the mother of polyenoic acids especially arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acids essential for the structure and function of cellular membranes in the developing fetus (1,2). Many studies have addressed the importance of the changes in long-chain fatty acid composition and metabolism that occur during fetal and neonatal development with respect to neonatal brain and somatic development (3–8). However, it is still unknown where within the maternal organism the essential fatty acids (EFA) used by the developing fetus and neonate do originate. Also unknown are the relative contributions of maternal EFA pools, *e.g.*, phospholipids, triacylglycerols (TG) or free fatty acids, to the EFA acquired by the fetus.

There is evidence that the maternal hepatic triacylglycerol pool may be quantitatively important in supplying EFA to the developing fetus (8). Since linoleic acid (18:2n-6) has several important functions, including as energy substrate (9), main dietary precursor of 20:4n-6, and as structural component of membranes, we have extended our initial study of EFA changes during pregnancy (8) by examining the changes in composition of maternal liver TG species late in pregnancy when the fetus is developing rapidly. We hypothesized that, as previously shown in fasted rats (10), identification of the TG class(es) (monolinoleoyl, dilinoleoyl, trilinoleoyl glycerols) may provide insight into the functional role of 18:2n-6, whether structural or as an energy substrate, during late pregnancy.

MATERIALS AND METHODS

Second parity pregnant rats (Sprague-Dawley) were obtained from Charles River (St. Constant, Quebec, Canada) at the earliest day of pregnancy possible (day 8). To provide known and constant fatty acid intake, they were given a semi-purified diet and deionized distilled water from day 8 until day 18 or term (day 21). The period between day 18 and day 21 was studied because it is when most of the changes in 18:2n-6 occur in maternal hepatic TG and also because it is when most fetal growth occurs in the rat. This period is therefore of greatest interest with respect to possible changes in maternal liver TG species. Animals killed on day 8 and did not consume the diet fed during pregnancy; hence, data for day 8 serve only as a reference. The semi-purified diet was prepared according to American Institute of Nutrition standards (components from Teklad Test Diets, Madison, WI) and contained (g/kg): 605, corn starch; 200, casein; 100, cellulose; 50, soybean oil (51% linoleic acid, 18:2n-6, and 7% linolenic acid, 18:3n-3); 35, Teklad modified AIN-76 mineral mix; and 10, AIN-76 vitamin mix.

All sovents used for lipid analysis were glass-redistilled prior to use. Maternal livers were removed and washed in saline and, prior to lipid analysis, 500 mg aliquots were kept in chloroform containing 0.02% butylated hydroxytoluene (BHT) (Sigma, St. Louis, MO) at −20°C. The total lipids were extracted from liver samples using chloroform/methanol (2:1, v/v) containing a final concentration of 0.02% BHT. To quantitate the individual TG species, 1,2-distearoyl-3-palmitoyl-glycerol (SSP) (Sigma), which was found to be naturally undetectable, was added to an aliquot of each sample as an internal standard (10). Total liver TG was isolated by thin-layer chromatography (TLC) with the solvent system of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). The TG band was recovered and eluted from the silica with 60 mL of hexane/diethyl ether (90:10, v/v). The TG species were analyzed using a Varian 3700 gas-liquid chromatograph (GLC) (Palo Alto, CA) equipped

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Abbreviations: BHT, butylated hydroxytoluene; C, triacylglycerol total acyl carbon number; EFA, essential fatty acid(s); GLC, gas-liquid chromatography; TG, triacylglycerol; TLC, thin-layer chromatography.

Abbreviations for triacylglycerol species are as follows: LLL, trilinoleoyl glycerol; MLO, myristoyl linoleoyl oleoyl glycerol; MPO, myristoyl palmitoyl oleoyl glycerol; MSP, myristoyl stearoyl palmitoyl glycerol; OLL, dilinoleoyl oleoyl glycerol; OLO, dioleoyl linoleoyl glycerol; OOO, trioleoyl glycerol; PLL, dilinoleoyl palmitoyl glycerol; PLO, palmitoyl linoleoyl oleoyl glycerol; PLP, dipalmitoyl linoleoyl glycerol; PLPo, palmitoyl linoleoyl palmitoleoyl glycerol; POO, dioleoyl palmitoyl glycerol; PoOO, dioleoyl palmitoleoyl glycerol; POP, dipalmitoyl oleoyl glycerol; POPo, palmitoyl oleoyl palmitoleoyl glycerol; POS, palmitoyl oleoyl stearoyl glycerol; PPP, tripalmitoyl glycerol; PPoPo, dipalmitoleoyl palmitoyl glycerol; PPPo, dipalmitoyl palmitoleoyl glycerol; PSL, palmitoyl stearoyl linoleoyl glycerol; PSP, dipalmitoyl stearoyl glycerol; PSPo, palmitoyl palmitoleoyl stearoyl glycerol; SLL, dilinoleoyl stearoyl glycerol; SLO, stearoyl linoleoyl oleoyl glycerol, SOO, dioleoyl stearoyl glycerol; SSL, distearoyl linoleoyl glycerol; SSP; distearoyl palmitoyl glycerol. (The sequence of letters used does not imply a specific positional order.)

with a TG column (25 m × 0.25 mm) coated with 65% phenylmethyl silicone film (Quadrex, New Haven, CT). The operating temperature was increased at 2°C/min from 320–355°C. A movable cold on-column injector (J & W Scientific, Folsom, CA) was used to permit injection of the sample at room temperature followed by rapid vaporization of the entire sample at the initial oven temperature (320°C), thus avoiding sample discrimination between TG species of different molecular weights (10,11). Peak areas were integrated using a Hewlett-Packard 3390A integrator (Palo Alto, CA), and total TG were quantitated based on the amount of standard added. The data for C52 and C54 TG species is not corrected for partial recovery of these species (see Results).

An aliquot of the total TG was hydrogenated at 70°C in a small conical vial. The TG sample was dissolved in 300 μL of hexane and transferred to the hydrogenation vial followed by addition of 1 mg of 5% rhodium on alumina powder (Aldrich Chemical Co., Milwaukee, WI) as a catalyst. The TG sample was bubbled with hydrogen gas for 5 min. Hexane (200 μL/min) was added to compensate for loss of solvent by evaporation. After hydrogenation was complete (verified by GLC of fatty acid methyl esters; ref. 10), the hexane layer was saved for separation of TG species according to total carbon number as described above.

An aliquot of the total lipid extract was used to analyze the fatty acid composition of liver TG. After TLC separation, the TG band was recovered and the fatty acids were converted to the corresponding methyl esters using 14% boron trifluoride in methanol (Sigma). Fatty acid methyl esters were analyzed by GLC using a capillary column (Durabond 225, 30 m × 25 mm, i.d.) coated with 25 μm cyanopropylphenyl (J & W Scientific) in a Hewlett-Packard 5890A GLC (Palo Alto) with automated sample delivery and injection (Hewlett-Packard 7671A) and peak integration (Hewlett-Packard 3393 integrator). The column temperature was programmed from 150°C to 220°C in three stages with each run complete within 30 min (8).

All data are expressed as the mean ± SD. Statistical comparison of data for day 18 and 21 was by Student's t-test.

RESULTS

Data are shown for day 8 (start of study), 18 and 21 of pregnancy. Emphasis is on the changes during days 18 to 21 when dietary fatty acid composition was constant. Liver weight increased 11% between days 18 and 21 (ns). During the same period, total liver TG increased by 53% (mg/g) or 69% (mg/liver; both $p<0.01$, Fig. 1).

Total non-EFA and total n-6 EFA in the maternal TG pool quantitatively increased while total n-3 EFA remained unchanged at day 21 compared to day 18. Palmitic (16:0), oleic (18:1n-9), linoleic (18:2n-6), eicosatrienoic (20:3n-6) and eicosapentaenoic (20:5n-3) acids increased quantitatively from day 18 to 21, while other fatty acids did not change (Table 1).

Individual TG species were identified by reference to standards, from the known fatty acid composition of the sample, and by reference to previous work (12,13). With the column used, resolution of TG species is on the basis of their total acyl carbon number (C) and total number of double bonds. Thus, overlapping of TG species with the same molecular weight and degree of unsaturation will occur; for example, dioleoyl palmitoyl glycerol and palmitoyl stearoyl linoleoyl glycerol overlap. As has been previously reported, liver TG with C >52 showed recoveries of less than 100% (10,12). This is consistent with the improved recovery of C > 54 after hydrogenation; however, information on individual species is thus lost (Fig. 2). Some individual TG species were identified; they are named by combining the first letter of the three fatty acid moieties, but not necessarily in specific positional order. These fractions may also include other species with the same total number of acyl carbons and double bands.

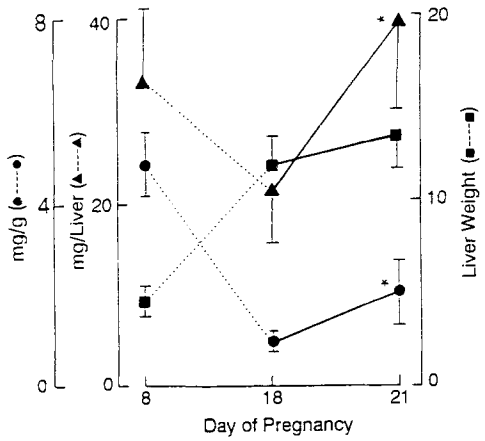

FIG. 1. Changes in rat liver weight (g, ■) and liver triacylglycerol content (mg/g, ●; mg/liver, ▲) from day 8 to day 21 of pregnancy (n = 6/group; mean ± SD). *p<0.01 in contrast to day 18.

TABLE 1

Maternal Liver Triacylglycerol Fatty Acid Composition (μg/g wet liver weight) in the Rat During Pregnancy (n = 6/time point; mean ± SD)

Fatty acid	Day 8	Day 18	Day 21	% Change[a]
16:0	955 ± 231	453 ± 73	612 ± 66	+35[b]
16:1n-7	63 ± 12	58 ± 28	77 ± 26	+33
18:0	143 ± 47	107 ± 32	146 ± 38	+36
18:1n-9	764 ± 135	484 ± 68	794 ± 48	+54[b]
18:2n-6	1831 ± 214	477 ± 58	791 ± 76	+66[b]
20:3n-6	22 ± 3	9 ± 1	19 ± 2	+111[b]
20:4n-6	282 ± 24	61 ± 17	84 ± 25	+38
22:4n-6	25 ± 4	tr[c]	tr	—
22:5n-6	9 ± 5	tr	tr	—
18:3n-3	167 ± 22	45 ± 10	59 ± 9	+31
20:5n-3	175 ± 51	10 ± 2	19 ± 2	+90[b]
22:5n-3	76 ± 5	17 ± 1	tr	−100
22:6n-3	471 ± 17	32 ± 10	25 ± 10	−22
Non EFA[d]	1925	1102	1629	+48[b]
n-6 EFA[e]	2169	547	894	+63[b]
n-3 EFA[f]	889	104	103	−1

[a] Compared to day 18.

[b] P< 0.01, in contrast to day 18.

[c] Trace amount.

[d] Sum of 16:0, 16:1n-7, 18:0 and 18:1n-9.

[e] Sum of 18:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6.

[f] Sum of 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3.

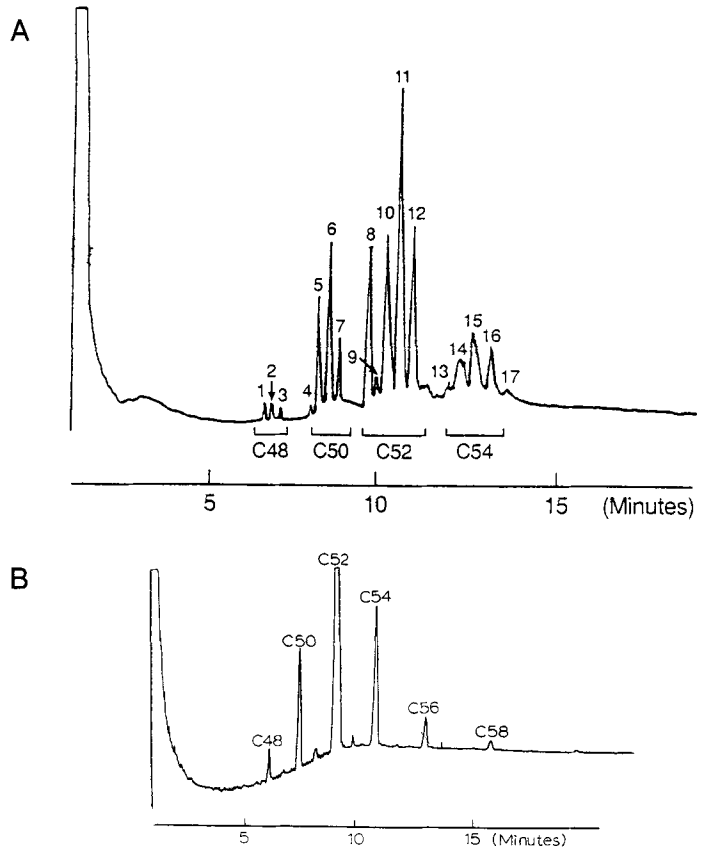

FIG. 2. A: Gas-liquid chromatogram of rat liver triacylglycerol species at day 21 of pregnancy. Column: fused silica capillary (25 m × 0.25 mm, i.d.) coated with 65% phenylmethyl silicone. Temperature: 320°C–355°C at 2°C/min. Carrier gas: Hydrogen (15 psi). Peak identification (including alternate species in brackets): 1, PPP (MSP); 2, PPPo (MPO); 3, PPoPo; 4, PSP; 5, POP (PSPo); 6, PLP (POPo); 7, PLPo (MLO); 8, SSP, internal standard; 9, POS; 10, POO (PSL); 11, PLO (PoOO); 12, PLL; 13, SOO (SSL); 14, SLO (OOO); 15, OLO (SLL); 16, OLL; 17, LLL; where P, palmitic acid; Po, palmitoleic acid; S, stearic acid; O, oleic acid; and L, linoleic acid. B: Gas-liquid chromatogram of hydrogenated rat liver triacylglycerol species (C, total acyl carbon number) from day 21 of pregnancy.

Between days 18 and 21, total C48 decreased, and C52–C58 increased, while C50 and C60 remained unchanged, relative to what was observed on day 18. Amounts of TG species >C52 are not corrected for recoveries, and are therefore only compared across time, not across TG species. 18:2n-6 represented more than 30% of total fatty acids in maternal liver TG and increased by 66% between days 18 and 21 ($p<0.01$). Consistent with the increase in 18:2n-6 in liver TG between day 18 and day 21, monolinoleoyl TG species increased: C50:3 (mainly PLPo), +47%; C52:3 (mainly PLO), +58%; C54:4 (mainly OLO), +67%, while C50:2 (mainly PLP) was unchanged. Dilinoleoyl species were also increased: C52:4 (mainly PLL), +155%, and C54:5 (mainly OLL), +96%. Trilinolein (C54:6) increased 429% from day 18 to 21. The other TG species did not change significantly except for tripalmitoyl glycerol, which decreased by 38% between day 18 and day 21 (Table 2, $p<0.05$).

Although data of day 8 and day 18 are not strictly comparable, total maternal liver TG at day 18 was significantly lower than at day 8 (mg/g or mg/liver; Fig. 1) with a proportional decrease over this period in n-6 and n-3 EFA, and a proportional increase in non-EFA; this is in agreement with our previous data (8). Based on the analyses of the hydrogenated TG species on day 18 compared to day 8, C48–C50 were significantly increased while C52-C60 were decreased (Table 2). Between days 8 and 18–21, when the rats were consuming twice as much linoleic acid/g diet as before day 8, TG species with one, two or three linoleoyl moieties were significantly decreased in maternal liver (μg/g).

TABLE 2

Maternal Liver Triacylglycerol Species Composition (μg/g wet liver weight) in the Rat During Pregnancy (n = 6/time point; mean ± SD)

Peak[a]	TG[b]	Day 8	Day 18	Day 21	% Change[c]
	C48	34 ± 3	98 ± 7	59 ± 14	−40[d]
1	PPP (MSP)	23 ± 2	37 ± 4	23 ± 6	−38[e]
2	PPPo (MPO)	6 ± 3	41 ± 15	24 ± 4	−41
3	PPoPo	5 ± 2	20 ± 5	12 ± 5	−40
	C50	276 ± 36	501 ± 76	447 ± 76	−14
4	PSP	12 ± 5	35 ± 10	11 ± 7	−69
5	POP (PSPo)	65 ± 13	217 ± 56	149 ± 42	−31
6	PLP (POPo)	120 ± 24	179 ± 15	174 ± 22	−3
7	PLPo (MLO)	79 ± 5	70 ± 13	103 ± 10	+47[e]
	C52	2139 ± 261	993 ± 43	1481 ± 55	+59[d]
9	POS	27 ± 8	39 ± 12	48 ± 18	+23
10	POO (PSL)	294 ± 34	446 ± 65	523 ± 113	+17
11	PLO (PoOO)	949 ± 44	399 ± 88	632 ± 103	+58[d]
12	PLL	869 ± 50	109 ± 20	278 ± 63	+155[d]
	C54	1408 ± 69	221 ± 47	571 ± 144	+158[d]
13	SOO (SSL)	64 ± 12	15 ± 7	22 ± 11	+46
14	SLO (OOO)	192 ± 40	34 ± 9	188 ± 58	+453[d]
15	OLO (SLL)	447 ± 97	98 ± 27	164 ± 29	+67[e]
16	OLL	554 ± 99	57 ± 19	112 ± 28	+96[d]
17	LLL	150 ± 14	17 ± 7	90 ± 13	+429[d]
	C56	949 ± 154	47 ± 16	167 ± 30	+252[d]
	C58	342 ± 86	12 ± 2	19 ± 2	+158[e]
	C60	78 ± 13	tr[f]	tr	

[a] See Figure 2 for identification.

[b] Triacylglycerol species; alternate species in brackets.

[c] Percent change in contrast to day 18.

[d] $P<0.01$ in contrast to day 18.

[e] $P<0.05$ in contrast to day 18.

[f] Trace amount.

DISCUSSION

Our data indicate that in the rat late in pregnancy (day 18 to 21-term), the composition of long-chain fatty acids and TG species in the total maternal liver TG pool is significantly altered, probably reflecting changing fatty acid requirements of the developing fetus or anticipation of lactation. The net changes reflect significant quantitative increases in non-EFA and n-6 EFA with no overall change in n-3 EFA. With the provision that recoveries are incomplete at >C52 and such species can only be compared across time, our data provide preliminary evidence that most of the quantitative increase in long-chain fatty acids in maternal liver TG was related to changes in 18:2n-6. Thus, although 16:0 and 18:1n-9 in maternal liver TG were increased 160 and 310 μg/g, respectively, between

day 18 and 21 of pregnancy, most of this increase could be accounted for by changes in the 16:0 and 18:1n-9 content of TG species also containing 18:2n-6 with no significant change or a decrease occurring in non-linoleoyl TG species (Table 2). Thus, 18:2n-6 appears to play a role in maternal liver TG of facilitating the accumulation of both 16:0 and 18:1n-9 during late pregnancy.

Total n-3 and n-6 EFA (μg/g) in the maternal hepatic TG pool increased markedly late in pregnancy and/or early in lactation with the highest values being observed at day 3 of lactation (4,8). These changes in maternal liver TG composition occur simultaneously with a dramatic increase in (4–10 fold) total n-3 and n-6 EFA in neonatal carcass or liver TG pools (14). Thus, the increase in the total linoleic acid-enriched TG pool in maternal liver seems to be an adaptation, or a reservoir, protecting against low maternal intake of linoleic acid during late pregnancy and early lactation.

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Alteration in Mouse Splenic Phospholipid Fatty Acid Composition and Lymphoid Cell Populations by Dietary Fat

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The fatty acid composition of diacyl- and alkylacyl-glycerophosphocholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), alkenylacyl-glycerophosphoethanolamine (aPE), and diacyl- and alkylacyl-glycerophosphoethanolamine (dPE) was assessed in isolated splenocytes from C3H/Hen mice fed one of four purified isocaloric diets for six weeks. Diets contained 20% by weight of either a high-linoleate sunflower oil (Hi 18:2), a high-oleate sunflower oil (Hi 18:1), a mixture of 17% menhaden fish oil and 3% high-linoleate sunflower oil (Hi n-3), or a mixture of 17% coconut oil and 3% high-linoleate sunflower oil (Hi SFA). Spleen weight and immune cell yield were significantly higher ($P < 0.05$) in mice fed the Hi 18:1 or the Hi n-3 diets compared with those fed the Hi 18:2 and Hi SFA diets. Distinctive patterns of fatty acids were observed for each phospholipid in response to dietary fatty acids. Dietary fat significantly affected ($P < 0.05$) total polyunsaturated fatty acids (PUFA) in PC and dPE, total saturated fatty acids (SFA) in PC, total monounsaturated fatty acids (MUFA), and n-3 PUFA in all phospholipid classes examined. In mice fed the Hi n-3 diet, n-3 PUFA were significantly elevated, whereas n-6 PUFA decreased in all of the phospholipids. In these mice, eicosapentaenoic acid (EPA) was the predominant n-3 PUFA in PC and PI, whereas docosahexaenoic acid (DHA) was the major n-3 PUFA in aPE and PS. Interestingly, the ratios of n-3/n-6 PUFA in the phospholipids from these mice were 3.2, 2.4, 1.8, 0.8 and 0.8 for aPE, PS, dPE, PC and PI, respectively. These data suggest a preferential incorporation of n-3 PUFA into aPE, PS and dPE over PC and PI.
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Phospholipids are the major structural components of cellular membranes and are involved in a range of biological responses including signal transduction and second messenger generation. It has been shown that the fatty acid composition of phospholipids can influence cell membrane fluidity, membrane-associated receptor and enzyme activities, as well as lipid mediator biosynthesis (1-7). Feeding animals diets which differ in fatty acid composition have been shown to influence cell membrane properties and biosynthesis of lipid mediators, such as eicosanoids and platelet-activating factor (7-10).

The spleen is a major site of immune response to blood-borne antigens, and it has been shown that dietary fat can significantly influence spleen immune cell function, such as antibody production and proliferation (11-14). Previously, Lokesh *et al.* (15) examined the fatty acid composition of the major phospholipid classes from whole spleen homogenates from mice fed diets containing either menhaden fish oil or coconut oil. Little is known about the effect that dietary fat source has on the fatty acid composition of spleen leukocyte phospholipid classes and subclasses, although such data have been reported for several other immune tissues and cells, including peritoneal macrophages and peripheral blood neutrophils (16-18).

The objective of the present study was to determine the effect of feeding various fat sources on the fatty acid composition of diacyl- and alkylacyl-glycerophosphocholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), alkenylacyl-glycerophosphoethanolamine (aPE), and diacyl- and alkylacyl-glycerophosphoethanolamine (dPE) of murine spleen leukocyte membranes. The fats tested in this study were menhaden fish oil, which is rich in n-3 polyunsaturated fatty acids (PUFA), a high-linoleate sunflower oil, a high-oleate sunflower oil, and coconut oil, which is rich in saturated fatty acids. Thus a wide range of fatty acid compositions are represented in these diets. All diets contained adequate linoleic acid to prevent essential fatty acid deficiency.

MATERIALS AND METHODS

Animals and diets. Weanling female C3H/Hen mice (Office of Laboratory Animal Medicine, University of Missouri, Columbia, MO) were used throughout this study. Mice were housed in hanging wire stainless steel cages (2-3 mice per cage) in a room where temperature and humidity were kept constant (*i.e.,* 21-24°C and 50-60%, respectively); a diurnal light cycle of 12 hr was maintained. Upon arrival, mice were randomly allotted to one of the following four purified diets that contained 20% by weight of either: i) a high-linoleate sunflower oil (Hi 18:2); ii) a high-oleate sunflower oil (Hi 18:1); iii) a mixture of 17% menhaden fish oil and 3% high-linoleate sunflower oil (Hi n-3); and iv) a mixture of 17% coconut oil and 3% high-linoleate sunflower oil (Hi SFA). The addition of 3% high-linoleate sunflower oil to Hi n-3 and Hi SFA was to ensure meeting the requirement for essential fatty acids (Table 1). The purified diets were isocaloric and formulated according to AIN guidelines (19). The composition of the purified diets was as follows (g/100 g): casein, 20; DL-methionine, 0.3; corn starch, 20; dextrose, 29.3; fiber, 5; mineral mix (AIN-76), 4; vitamin mix (AIN-76), 1.2; choline bitartrate, 0.2; oil, 20. The fatty acid composition of the diets is shown in Table 1. Animals were given

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; GC, gas chromatography; Hi 18:1, high-oleate sunflower oil; Hi 18:2, high-linoleate sunflower oil; Hi n-3, a mixture of 17% menhaden fish oil and 3% high-linoleate sunflower oil; Hi SFA, a mixture of 17% coconut oil and 3% high-linoleate sunflower oil; HPTLC, high-performance thin-layer chromatography; MUFA, monounsaturated fatty acids; PC, diacyl- and alkylacyl-glycerophosphocholine; aPE, alkenylacyl-glycerophosphoethanolamine; dPE, diacyl- and alkylacyl-glycerophosphoethanolamine; PI, phosphatidylinositol; PMN, polymorphonuclear leukocytes; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TLC, thin-layer chromatography.

TABLE 1

Fatty Acid Composition of Diets

Fatty acids[b]	Diet[a] (wt %)			
	Hi 18:2	Hi 18:1	Hi n-3	Hi SFA
8:0	nd	nd	nd	3.6
10:0	nd	nd	nd	4.9
12:0	nd	nd	nd	40.4
14:0	1.0	tr	5.8	15.3
16:0	6.8	3.8	15.5	9.2
16:1n-7	tr	tr	7.7	0.1
18:0	5.0	4.5	3.5	4.3
18:1n-7 and 9	16.9	77.0	13.2	8.8
18:2n-6	67.9	12.0	12.3	13.1
18:3n-6	tr	tr	0.3	nd
18:3n-3 and 4n-3	0.4	0.3	4.4	tr
20:5n-3	0.7	1.0	11.5	nd
22:5n-3	0.2	0.3	1.7	nd
22:6n-3	nd	nd	10.2	nd
SFA (C_8-C_{18})	12.8	8.3	24.8	77.7
MUFA	17.0	77.1	20.9	8.9
Total n-3	1.3	1.6	29.2	tr
Total n-6	68.1	12.0	13.9	13.1
Total PUFA	69.4	13.6	43.1	13.1

[a] Hi 18:2 contained 20% linoleic sunflower oil (Beatrice/Hunt-Wesson, Inc., Fullerton, CA); Hi 18:1 contained 20% oleic sunflower oil (SVO Enterprises, Painesville, OH); Hi n-3 contained 17% menhaden fish oil (Zapata Haynie, Reedville, VA) plus 3% linoleic sunflower oil; Hi SFA contained 17% coconut oil (Karlshamns USA Inc., Harrison, NJ) plus 3% linoleic sunflower oil. All data are expressed as percentage (%) by weight.

[b] Fatty acids are denoted by the number of carbons; the number of double bonds, followed by the position of the first double bond relative to the terminal methyl group ("n-"). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; tr, trace (<0.2%); and nd, not detectable.

fresh diet every day, with any remaining diet being discarded, and were provided with tap water *ad libitum*. Autoxidation of diets was prevented by adding 0.02% *t*-butyl hydroquinone (Eastman Kodak Co., Rochester, NY) to the oils upon receipt. Diets were made up every two weeks. Peroxide values of extracted diet lipids were periodically checked and never exceeded 50 meq/kg.

Preparation of spleen cells. After six weeks of feeding mice the experimental diets, mice were anesthetized by intramuscular injection of 0.15 mL Ketaset (Aveco Co., Inc., Fort Dodge, IA) containing 8 mg/mL of Rompun (Mobay Co., Animal Health Division, Shawnee, KS). Spleens were removed, weighed and dispersed in 10 mL of RPMI 1640 medium by passage through a 80-mesh stainless steel screen. A single cell suspension was obtained by allowing tissue clumps to settle out at room temperature for 10 min. Cells were then sedimented by centrifugation (300 × *g*) at 20°C for 5 min. Erythrocytes were lysed with 1% NH_4Cl. After the sedimentation of lysed-cell debris for 3 min at room temperature, the cell suspension was centrifuged (300 × *g*) at 20°C for 5 min. The obtained leukocytes were washed twice and resuspended in RPMI 1640 and were enumerated using a Coulter Counter (Model ZIP, Coulter Electrics Inc., Hiaheah, FL). A small portion of cells was subjected to cytospin for 5 min at 800 rpm on a Shandon Cytospin (Boyce Scientific Inc., St. Louis, MO), and were stained with Hema-Tek Stain Pak using an Hema-Tek Slide Stainer (Ames Company, Division of Miles Laboratories, Elkhart, IN). Differential counts of spleen cells were done using a light microscope. The remaining cells were washed once with PBS and resuspended in 1 mL of 10 mM EDTA and stored at −20°C until analyzed for phospholipid fatty acid composition.

Phospholipid separation and fatty acid analysis. Total cell lipids were extracted as described by Sun (20). Briefly, to one volume of spleen leukocyte suspension, four volumes of chloroform/methanol (2:1, v/v) were added. The tubes were thoroughly mixed by vortexing, then centrifuged at 500 × *g* for 10 min at 10°C. The lower organic phase was removed and transferred to another test tube. The aqueous phase was subjected to a second extraction with 2 volumes of chloroform/methanol/12 N HCl (4:1:0.013, by vol). The acidic organic phase was transferred to another tube and neutralized with 1 drop of 16 N NH_4OH before combining with the first organic extract. It has been shown that by using this procedure, a complete extraction of both neutral and acidic phospholipids can be attained (20). The combined extract was filtered through anhydrous Na_2SO_4 and taken to dryness under N_2. Lipids were resuspended in chloroform/methanol (2:1, v/v) and applied to the left lower corner of high-performance thin-layer chromatography (HPTLC) silica gel 60 plates (E. Merck, Darmstadt, Germany) and developed in a solvent system containing chloroform/methanol/acetone/NH_4OH (35:20:5:5, by vol) for 30 min. After developing the plates, the solvent was removed by blowing a light stream of air over the plates for 10–15 min. The plates were then exposed to HCl fumes for 3 min to hydrolyze the plasmalogenic PE fraction to give lysoPE and aldehyde. After drying in an air stream for 10–15 min, the plates were turned 90° and placed in the second solvent system consisting of chloroform/methanol/acetone/acetic acid/0.1 M ammonium acetate (35:15:13.8:1.55:2.5, by vol) and developed for another 30 min. The TLC plates were then dried and sprayed with 2′,7′-dichlorofluorescein (0.1% in methanol). The lipid fractions were made visible under a UV lamp and scraped into 30-mL screw-capped tubes and closed with Teflon-lined caps. An appropriate amount of 17:0 fatty acid methyl ester was added as an internal standard. Fatty acid transmethylation was carried out by incubating with 1 mL of 0.5M NaOH per tube for 10 min, followed by 2 mL chloroform for another 10 min. Fatty acid methyl esters (FAME) were then extracted by adding 0.75 mL H_2O. The lower phases were then filtered through sodium sulfate, and FAME were analyzed using a Hewlett-Packard 5890 gas chromatograph (GC) (Hewlett-Packard, Norwalk, CT) equipped with a 30-m × 0.25-mm inside diameter fused silica capillary column (Supelco, Bellefonte, PA). Helium was used as carrier gas at a flow rate of 1 mL/min. Oven temperature was held at 190°C for 10 min, then increased at 3°C/min to 230°C, where it was maintained for 11.67 min. FAME were identified by comparing their relative retention times with those of the commercial standards PUFA-1 and PUFA-2 (Supelco).

Since fatty acyl groups of aPE are derived exclusively from the *sn*-2 position, whereas the fatty acyl groups of PC, PI, PS and dPE represent both *sn*-1 and *sn*-2 positions, the molar amounts of total FAME from aPE were

divided by one, while those from PC, PI, PS and dPE were divided by two for relative phospholipid class and subclass composition analysis as described by others (15). The data were expressed as mol% of total phospholipids or total FAME identified.

Statistical analysis. The data were subjected to one-way analysis of variance to test for the effect of the dietary fat source. When significant differences occurred ($P < 0.05$), Fisher's least significant difference was used for comparison of treatment means. Data analyses were processed on a Macintosh II computer using version 1.03 of StatView II (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Body weight, spleen weight and splenic cellularity. Feeding mice the experimental diets for six weeks resulted in an average body weight gain of 19.2 g. As shown in Table 2, there was no effect of dietary fat source on body weight gain. However, dietary fat source did have a significant influence on spleen weight, as well as the number of spleen leukocytes recovered ($P < 0.01$). Spleens from mice fed the Hi 18:1 and Hi n-3 diets were heavier and had more leukocytes than those from mice fed the Hi 18:2 and Hi SFA diets. There was no diet effect on the percentage of lymphocytes, macrophages, and polymorphonuclear leukocytes (PMN) found in the spleen. However, the total number of both lymphocyte and macrophages per spleen were significantly influenced by dietary fat source. Mice fed the Hi 18:1 and Hi n-3 diets had significantly more lymphocytes and macrophages than mice fed the Hi 18:2 and Hi SFA diets ($P < 0.05$).

Phospholipid composition of murine spleen leukocytes. The two-dimensional TLC procedure allowed for a separation of PI from PS, and plasmalogens from their respective diacyl phospholipids, but was unable to separate the alkylacyl phospholipid species from diacyl species (20). Therefore, PC contained both diacyl- and alkylacyl-glycerophosphocholine and dPE contained both diacyl- and alkylacyl-glycerophosphoethanolamine. Figure 1 shows the relative composition as mol% of leukocyte phospholipids examined. As shown in the Figure, the dietary fat source did not alter the phospholipid composition of murine splenic leukocytes. PC was the most abundant phospholipid in the cell membrane and it accounted for about 60% of the total phospholipids examined, followed by dPE, which was about 20% of total phospholipids. PI, PS and aPE each accounted for only about 6–8% of total phospholipids tested.

Fatty acid composition of murine leukocyte diacyl- and alkylacyl-glycerophosphocholine (PC). Table 3 shows the fatty acid composition of PC from murine splenic leukocytes. SFA, MUFA and n-3 PUFA, as well as linoleic acid (18:2n-6) in PC, were reflective of dietary fatty acids. In mice fed the Hi n-3 diet, an elevation of n-3 PUFA, predominantly eicosapentaenoic acid (EPA, 20:5n-3) and, to a lesser extent, docosahexaenoic acid (DHA, 22:6n-3) and docosapentaenoic acid (DPA, 22:5n-3), were observed in leukocyte PC. A small but significant increase of AA was observed in mice fed the Hi SFA diet as compared to mice fed the Hi 18:2 and Hi 18:1 diets. Total n-6 PUFA were significantly altered by dietary fat source (Hi 18:2 > Hi SFA > Hi 18:1 > Hi n-3, $P < 0.05$). Total PUFA in PC were also significantly affected by dietary fat source

TABLE 2

Effect of Dietary Fat on Spleen Weight and Spleen Cell Populations of Mice[a]

Parameters	Diet			
	Hi 18:2	Hi 18:1	Hi n-3	Hi SFA
Initial body weight (g)	12.2 ± 0.4	12.4 ± 0.3	12.2 ± 0.4	12.2 ± 0.4
Final body weight (g)	30.9 ± 0.5	32.5 ± 0.7	31.3 ± 0.7	31.2 ± 0.6
Spleen weight (mg)	126.0 ± 5.7[f]	161.7 ± 9.2[e]	160.3 ± 6.0[e]	128.9 ± 6.5[f]
Leukocytes per spleen[b]	8.8 ± 0.6[f]	12.1 ± 0.7[e]	11.6 ± 0.6[e]	8.7 ± 0.4[f]
Lymphocytes				
Number per spleen[b]	6.9 ± 0.5[f]	9.4 ± 0.5[e]	9.0 ± 0.5[e]	6.9 ± 0.3[f]
Percentage (%)	78.0 ± 2.0	77.7 ± 1.6	77.4 ± 2.0	80.7 ± 2.1
Macrophages				
Number per spleen[b]	1.1 ± 0.1[f]	1.6 ± 0.1[e]	1.5 ± 0.1[e]	1.0 ± 0.1[f]
Percentage (%)	12.6 ± 1.1	13.5 ± 0.8	12.7 ± 0.9	11.0 ± 1.1
PMN[c]				
Number per spleen[b]	0.6 ± 0.06	0.6 ± 0.09	0.7 ± 0.12	0.5 ± 0.14
Percentage (%)	6.5 ± 0.8	5.5 ± 0.8	6.6 ± 1.2	5.5 ± 1.2
Other cells[d]				
Number per spleen[b]	0.3 ± 0.04	0.4 ± 0.06	0.4 ± 0.04	0.2 ± 0.04
Percentage (%)	2.9 ± 0.4	3.2 ± 0.4	3.2 ± 0.3	2.8 ± 0.4

[a] Three separate experiments (N=9 per treatment) were conducted with three-week-old female C3H/Hen mice. Mice were fed the experimental diets for six weeks and spleens were obtained for separating leukocytes as described in the Methods section. Cell types were characterized by differential count from cytospin staining. Values represent mean and standard error of mean (SEM); values within each row which do not share a common letter (e,f) are statistically different at $P < 0.05$.

[b] Cell numbers ($\times 10^7$).

[c] PMN, polymorphonuclear leukocytes.

[d] Other cells were those cells that were neither lymphocytes, macrophages, nor PMN.

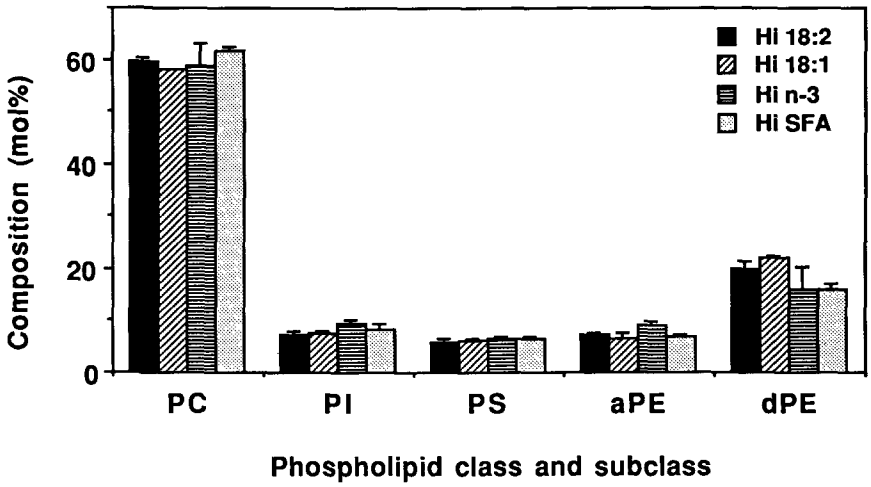

FIG. 1. Phospholipid composition of murine spleen leukocytes. Results represent mean ± SEM (n=3). Values are not significantly different (P > 0.05).

TABLE 3

Effect of Dietary Fat on Fatty Acid Composition of Murine Spleen Leukocyte Diacyl- and Alkylacyl-glycerophosphocholine (PC)

Fatty acids	Diet (mol %)			
	Hi 18:2	Hi 18:1	Hi n-3	Hi SFA
14:0	0.6 ± 0.04^c	0.4 ± 0.03^c	2.0 ± 0.04^b	4.6 ± 0.05^a
16:0	45.3 ± 0.77^b	41.3 ± 1.02^c	49.8 ± 0.24^a	45.8 ± 0.88^b
16:1	0.4 ± 0.03^c	0.7 ± 0.24^c	3.1 ± 0.17^a	1.3 ± 0.17^b
18:0	13.7 ± 0.19^a	10.4 ± 0.27^c	10.3 ± 0.07^c	11.4 ± 0.31^b
18:1	8.8 ± 0.46^c	25.5 ± 0.44^a	12.0 ± 0.18^b	12.1 ± 0.57^b
18:2n-6	14.9 ± 0.25^a	4.0 ± 0.2^d	7.4 ± 0.05^b	6.3 ± 0.32^c
20:2n-6	1.3 ± 0.07^a	0.3 ± 0.01^c	0.2 ± 0.00^c	0.5 ± 0.02^b
20:3n-6	0.7 ± 0.02^a	0.5 ± 0.02^b	0.7 ± 0.05^a	0.7 ± 0.01^a
20:4n-6	11.0 ± 0.59^b	11.9 ± 0.59^b	3.8 ± 0.04^c	13.6 ± 0.59^a
20:5n-3	tr^b	0.2 ± 0.05^b	5.2 ± 0.14^a	tr^b
22:1n-9	0.7 ± 0.04^b	1.7 ± 0.39^a	0.5 ± 0.09^b	0.5 ± 0.28^b
22:4n-6	0.9 ± 0.07^a	1.0 ± 0.08^a	0.2 ± 0.03^b	1.1 ± 0.05^a
22:5n-6	0.7 ± 0.07^b	1.0 ± 0.11^a	0.3 ± 0.01^c	1.2 ± 0.11^a
22:5n-3	tr^b	0.2 ± 0.02^b	1.6 ± 0.02^a	0.2 ± 0.01^b
22:6n-3	0.6 ± 0.10^b	0.8 ± 0.08^b	2.9 ± 0.12^a	0.7 ± 0.13^b
SFA	59.6 ± 0.92^a	52.1 ± 0.92^b	62.0 ± 0.24^a	61.8 ± 1.62^a
MUFA	10.0 ± 0.47^c	27.8 ± 0.90^a	15.6 ± 0.33^b	13.8 ± 0.77^b
Total n-3 PUFA	0.7 ± 0.10^c	1.2 ± 0.03^b	9.7 ± 0.21^a	1.0 ± 0.15^{b,c}
Total n-6 PUFA	29.7 ± 1.03^a	18.8 ± 0.52^c	12.6 ± 0.07^d	23.4 ± 0.78^b
Total PUFA	30.4 ± 1.15^a	20.1 ± 0.53^c	22.3 ± 0.17^{b,c}	24.0 ± 0.87^b

$^{a-e}$All values are expressed as means (mol%) and pooled SEM. Means with different letter within the same fatty acid group are significantly different at P < 0.05; n=3 per treatment.

(P < 0.05), with mice fed the Hi 18:2 diet having the highest, and mice fed the Hi 18:1 diet having the lowest total PUFA.

Fatty acid composition of murine spleen leukocyte phosphatidylinositol (PI). Table 4 shows the fatty acid composition of PI from murine splenic leukocytes. Unlike PC, dietary fat source did not significantly affect percentage of SFA in membrane PI. In mice fed the Hi 18:1 diet, MUFA in PI were the highest as compared to other dietary fat sources. Linoleic acid level in PI of mice fed the Hi 18:2 diet was the highest compared to other diets. However, this level was only one fourth of that in PC. Feeding diets high in SFA and MUFA did not alter AA nor total PUFA level in PI when compared to high linoleate feeding. However, feeding the Hi n-3 diet reduced AA by almost 50% with a concomitant increase in EPA, which was the major n-3 PUFA in PI. Unlike in PC, total PUFA in PI were not affected by dietary fat source.

TABLE 4

Effect of Dietary Fat on Fatty Acid Content of Murine Spleen Leukocyte Phosphatidylinositol (PI)

Fatty acids	Diet (mol %)			
	Hi 18:2	Hi 18:1	Hi n-3	Hi SFA
16:0	6.4 ± 0.62	6.1 ± 1.47	9.8 ± 1.13	10.2 ± 1.87
16:1	1.1 ± 0.61	0.6 ± 0.38	0.3 ± 0.15	0.2 ± 0.2
18:0	58.0 ± 2.93	50.9 ± 2.37	51.8 ± 2.40	54.4 ± 3.68
18:1	3.3 ± 0.67^{c}	8.4 ± 0.44^{a}	5.6 ± 0.31^{b}	4.9 ± 1.07^{b,c}
18:2n-6	3.1 ± 0.19^{a}	1.1 ± 0.07^{c}	2.3 ± 0.11^{b}	1.4 ± 0.14^{c}
20:4n-6	23.3 ± 3.26^{a}	25.9 ± 1.95^{a}	13.2 ± 0.76^{b}	23.2 ± 2.56^{a}
20:5n-3	0.3 ± 0.30^{b}	0.9 ± 0.29^{b}	9.8 ± 1.00^{a}	tr^{b}
22:1n-9	0.5 ± 0.24	1.2 ± 0.32	0.3 ± 0.18	0.7 ± 0.16
22:4n-6	1.6 ± 0.31^{a}	1.5 ± 0.09^{a}	tr^{b}	1.4 ± 0.24^{a}
22:5n-6	1.7 ± 1.47	0.8 ± 0.11	tr	0.7 ± 0.13
22:5n-3	tr^{b}	0.5 ± 0.48^{b}	2.3 ± 0.16^{a}	tr^{b}
22:6n-3	0.4 ± 0.44^{b}	1.0 ± 0.08^{a,b}	2.6 ± 0.27^{a}	1.8 ± 0.83^{a,b}
SFA	64.4 ± 3.43	57.0 ± 2.12	61.7 ± 2.55	64.7 ± 3.15
MUFA	4.9 ± 0.49^{b}	10.2 ± 0.15^{a}	6.2 ± 0.64^{b}	5.8 ± 1.15^{b}
Total n-3 PUFA	0.7 ± 0.39^{b}	2.5 ± 0.23^{b}	14.7 ± 1.35^{a}	2.0 ± 0.76^{b}
Total n-6 PUFA	29.9 ± 2.94^{a}	30.3 ± 2.01^{a}	17.4 ± 0.77^{d}	27.5 ± 2.98^{a}
Total PUFA	30.7 ± 3.25	32.7 ± 2.23	32.1 ± 2.05	29.5 ± 2.35

$^{a-d}$All values are expressed as means (mol%) and pooled SEM. Means with different letter within the same fatty acid group are significantly different at $P < 0.05$; n=3 per treatment.

TABLE 5

Effect of Dietary Fat on Fatty Acid Content of Murine Spleen Leukocyte Phosphatidylserine (PS)

Fatty acids	Diet (mol %)			
	Hi 18:2	Hi 18:1	Hi n-3	Hi SFA
16:0	8.3 ± 1.17	8. ± 1.14	12.3 ± 1.50	10.9 ± 1.27
16:1	1.2 ± 1.19	0.9 ± 0.45	0.6 ± 0.31	tr
18:0	62.5 ± 7.33	55.5 ± 5.75	56.0 ± 6.23	57.8 ± 6.27
18:1	3.8 ± 0.78^{b}	10.4 ± 2.05^{a}	4.9 ± 1.13^{b}	5.4 ± 1.60^{b}
18:2n-6	5.5 ± 1.52	1.8 ± 0.40	3.4 ± 0.68	2.6 ± 0.65
20:4n-6	8.6 ± 3.19^{a}	9.2 ± 2.61^{a}	2. ± 0.76^{b}	9.9 ± 3.42^{a}
20:5n-3	0.5 ± 0.48^{b}	0.9 ± 0.23^{b}	3.6 ± 0.75^{a}	tr^{b}
22:2n-9	0.3 ± 0.25^{b}	2.4 ± 1.19^{a}	0.2 ± 0.16^{b}	0.4 ± 0.20^{b}
22:4n-6	3.2 ± 1.26^{a}	3.0 ± 0.99^{a}	0.2 ± 0.17^{b}	2.8 ± 0.96^{a}
22:5n-6	2.5 ± 0.91^{a}	3.3 ± 0.88^{a}	0.4 ± 0.18^{b}	3.9 ± 1.30^{a}
22:5n-3	tr^{b}	tr^{b}	3.7 ± 1.21^{a}	0.5 ± 0.36^{b}
22:6n-3	2.9 ± 0.36^{b}	3.1 ± 0.59^{b}	11.1 ± 2.88^{a}	4.7 ± 1.93^{b}
SFA	70.8 ± 8.41	64.0 ± 6.22	68.3 ± 7.72	68.7 ± 7.01
MUFA	5.2 ± 1.74^{b}	13.7 ± 0.67^{a}	5.6 ± 1.02^{b}	5.8 ± 1.80^{b}
Total n-3 PUFA	3.4 ± 0.17^{b}	4.1 ± 0.52^{b}	18.4 ± 4.85^{a}	5.2 ± 1.74^{b}
Total n-6 PUFA	20.6 ± 7.32^{a}	18.2 ± 5.29^{a}	7.7 ± 1.87^{b}	20.3 ± 6.79^{a}
Total PUFA	24.0 ± 7.19	22.3 ± 5.76	26.0 ± 6.71	25.5 ± 5.27

^{a,b}All values are expressed as means (mol%) and pooled SEM. Means with different letter within the same fatty acid group are significantly different at $P < 0.05$; n=3 per treatment.

Fatty acid composition of murine spleen leukocyte phosphatidylserine (PS). Table 5 shows the fatty acid composition of PS from murine splenic leukocytes. Unlike PC, but similar to PI, leukocyte PS contained similar amounts of SFA for all the four dietary treatments. Similar to PI, stearic acid (18:0) was the predominant fatty acid present in spleen leukocyte PS and accounted for over 55% of the total fatty acids present. The amount of oleic acid (18:1) in PS from mice fed Hi 18:1 diet was similar in magnitude as that observed for PC and PI. Hi n-3 feeding caused an elevation of EPA, DPA and DHA with a concomitant reduction in AA and other longer chain n-6 PUFA. Unlike PC and PI, where EPA was the major n-3 PUFA, PS contained predominantly DHA as the major n-3 PUFA. Consumption of a diet rich in SFA and MUFA did not alter AA nor total n-6 PUFA levels in PS when compared to a diet rich in linoleate (Hi 18:2). Total PUFA levels in PS were not affected by dietary fat source.

TABLE 6

Effect of Dietary Fat on Fatty Acid Content of Murine Spleen Leukocyte Alkenylacyl-glycerophosphoethanolamine (aPE)

Fatty acids	Diet (mol %)			
	Hi 18:2	Hi 18:1	Hi n-3	Hi SFA
16:0	9.3 ± 2.45	7.9 ± 0.70	7.4 ± 2.60	8.6 ± 2.42
16:1	0.8 ± 0.78	0.3 ± 0.24	0.8 ± 0.40	1.0 ± 1.07
18:0	9.3 ± 4.34	5.3 ± 1.07	9.5 ± 3.44	9.8 ± 3.24
18:1	2.7 ± 0.76^b	7.4 ± 1.72^a	3.1 ± 0.09^b	5.4 ± 1.81^{a,b}
18:2n-6	6.7 ± 0.18^a	3.1 ± 0.21^b	4.1 ± 0.44^b	3.1 ± 0.45^b
20:4n-6	40.4 ± 3.39^a	40.6 ± 1.70^a	13.0 ± 0.70^b	38.6 ± 4.07
20:5n-3	tr^b	0.3 ± 0.35^b	16.9 ± 1.09^a	tr^b
22:1n-9	tr^b	1.7 ± 0.47^a	tr^b	0.6 ± 0.65^b
22:4n-6	15.8 ± 1.94^a	14.2 ± 0.23^a	1.0 ± 0.06^b	13.9 ± 1.28^a
22:5n-6	7.7 ± 1.12^a	9.6 ± 0.84^a	0.7 ± 0.36^b	11.5 ± 2.08^a
22:5n-3	0.8 ± 0.41^b	1.2 ± 0.23^b	12.8 ± 1.24^a	0.3 ± 0.26^b
22:6n-3	6.6 ± 0.21^b	8.3 ± 0.72^b	30.4 ± 2.91^a	7.3 ± 0.08^b
SFA	18.6 ± 6.79	13.2 ± 0.46	17.2 ± 6.38	18.4 ± 5.67
MUFA	3.5 ± 0.76^b	9.3 ± 2.32^a	3.9 ± 0.43^b	7.1 ± 2.11^{a,b}
Total n-3 PUFA	7.4 ± 0.55^b	9.9 ± 1.00^b	60.0 ± 5.26^a	7.5 ± 0.30^b
Total n-6 PUFA	70.5 ± 6.61^a	67.5 ± 2.93^a	18.8 ± 1.52^b	67.0 ± 7.90^a
Total PUFA	77.9 ± 7.14	77.4 ± 1.89	78.9 ± 6.74	74.6 ± 7.62

^{a,b}All values are expressed as mean (mol%) and pooled SEM. Means with different letter within the same fatty acid group are significantly different at $P < 0.05$; n=3 per treatment.

Fatty acid composition of splenic leukocyte alkenylacyl-glycerophosphoethanolamine (aPE). Table 6 shows the fatty acid composition of aPE from murine splenic leukocytes. Splenocyte aPE contained less SFA and much higher amounts of PUFA as compared to the other phospholipids examined. Dietary fat source did not influence SFA level in aPE. The effect of dietary fat on MUFA in splenocyte aPE were similar to those observed in other phospholipids with total MUFA being the highest in mice fed the Hi 18:1 diet. Hi n-3 feeding caused an elevation of EPA, DPA and DHA with a concomitant reduction in AA and other longer chain of n-6 PUFA. Similar to PS, aPE contained predominantly DHA as the major n-3 PUFA. In addition, consumption of a diet rich in SFA and MUFA did not alter AA nor total n-6 PUFA levels in aPE when compared to a diet rich in linoleate (Hi 18:2). Total PUFA levels in aPE were not affected by dietary fat source.

Fatty acid composition of diacyl- and alkylacyl-glycerophosphoethanolamine (dPE) of murine splenic leukocytes. Table 7 shows the fatty acid composition of dPE from murine splenic leukocytes. Dietary fat source significantly affected the percentage of palmitic acid (16:0) and stearic acid (18:0) in dPE. However, there was no diet effect on total SFA. The pattern of MUFA in leukocyte dPE were similar as in other phospholipids with MUFA being the highest in mice fed the Hi 18:1 diet. Hi n-3 feeding caused an elevation of EPA, DPA and DHA with a concomitant reduction in AA and other longer chain of n-6 PUFA. Unlike other phospholipids, dPE contained both DHA and EPA as the predominant n-3 PUFA (12.4% *vs.* 10.0% for DHA and EPA, respectively, $P < 0.05$). The effect of fat source on AA and total n-6 PUFA in dPE was very similar to that noted in PC. A small but statistical significant increase of AA was also observed in mice fed the Hi SFA diet as compared to mice fed the Hi 18:2 or Hi 18:1 diets. Total n-6 PUFA were significantly altered ($P < 0.05$) by dietary fat source (Hi 18:2 > Hi SFA > Hi 18:1 > Hi n-3). Total PUFA in dPE were also significantly affected by dietary fat source ($P < 0.05$) with mice fed Hi 18:2 diet having the highest and mice fed Hi 18:1 having the lowest total PUFA.

The n-3/n-6 PUFA ratio of murine splenic leukocyte phospholipids. Figure 2 shows the effect of dietary fat on phospholipid n-3 PUFA/n-6 PUFA ratio. As shown in the Figure, the n-3/n-6 ratio in every phospholipid examined was significantly higher ($P < 0.0001$) in mice fed Hi n-3 diet than mice fed other diets. Interestingly, the n-3/n-6 ratio in mice fed Hi n-3 diet was significantly different among the five phospholipids (aPE > PS > dPE > PI and PC, $P < 0.05$).

DISCUSSION

In the present investigation, we have demonstrated that the source of dietary fat provided to mice produces marked changes in the fatty acid composition of their splenic leukocyte membrane phospholipids. Furthermore, these data show that the effects were unique for each phospholipid. Similar results have been observed by others (15,17, 18,21-23) who studied the effect of dietary fat source on fatty acid composition of whole spleen or splenocyte phosphatidylcholine and phosphatidylethanolamine in rats or mice. These data indicate that there is a marked difference in fatty acid composition between these two classes of phospholipids in the spleen of mice or rats fed diets containing different fats. We have shown that the differences in fatty acid composition induced by dietary fats were observed not only between classes, but also between subclasses of phospholipids. Others have observed

TABLE 7

Effect of Dietary Fat on Fatty Acid Content of Murine Spleen Leukocyte Diacyl- and Alkylacyl-glycerophosphoethanolamine (dPE)[a]

Fatty acids	Diet (mol %)			
	Hi 18:2	Hi 18:1	Hi n-3	Hi SFA
16:0	11.1 ± 0.09[b]	11.5 ± 1.84[b]	16.9 ± 0.17[a]	13.3 ± 1.04[b]
16:1	tr[c]	tr[c]	1.4 ± 0.09[a]	0.5 ± 0.23[b]
18:0	36.1 ± 0.73[a]	29.5 ± 1.58[b]	29.1 ± 1.32[b]	32.2 ± 0.10[b]
18:1	8.3 ± 0.54[b]	20.8 ± 4.43[a]	10.5 ± 0.17[b]	13.1 ± 3.12[b]
18:2n-6	10.2 ± 0.41[a]	3.9 ± 0.03[b,c]	4.9 ± 0.19[b]	3.2 ± 0.49[c]
20:2n-6	1.1 ± 0.11[a]	0.3 ± 0.01[b]	tr[b]	0.3 ± 0.04[b]
20:3n-6	0.7 ± 0.01	0.5 ± 0.03	0.8 ± 0.21	0.5 ± 0.04
20:4n-6	21.5 ± 0.53[b]	21.5 ± 0.93[b]	7.8 ± 0.09[c]	23.9 ± 0.83[a]
20:5n-3	0.6 ± 0.03[b]	0.4 ± 0.04[b]	10.0 ± 0.19[a]	tr[c]
22:1n-9	tr[c]	0.9 ± 0.17[a]	0.4 ± 0.02[b]	0.9 ± 0.13[a]
22:4n-6	4.6 ± 0.07[a]	4.0 ± 0.05[b]	0.2 ± 0.11[c]	4.5 ± 0.06[a]
22:5n-6	3.1 ± 0.10[b]	3.5 ± 0.39[b]	0.6 ± 0.04[c]	5.0 ± 0.59[a]
22:5n-3	0.2 ± 0.01[b]	0.3 ± 0.02[b]	4.7 ± 0.18[a]	0.3 ± 0.03[b]
22:6n-3	1.9 ± 0.15[b]	2.4 ± 0.16[b]	12.4 ± 0.56[a]	2.3 ± 0.20[b]
SFA	47.5 ± 0.67	41.4 ± 3.45	46.0 ± 1.21	45.7 ± 1.13
MUFA	8.9 ± 0.51[b]	21.6 ± 4.62[a]	12.3 ± 0.12[b]	14.4 ± 3.00[a,b]
Total n-3 PUFA	2.2 ± 0.15[b]	3.1 ± 0.21[b]	27.0 ± 0.91[a]	2.6 ± 0.19[b]
Total n-6 PUFA	41.5 ± 1.35[a]	33.9 ± 1.37[b]	14.8 ± 0.38[c]	37.3 ± 2.07[a,b]
Total PUFA	43.7 ± 1.19[a]	37.0 ± 1.17[b]	41.7 ± 1.33[a]	39.9 ± 1.89[a,b]

[a-c]All values are expressed as means (mol%) and pooled SEM. Means with different letter within the same fatty acid group are significantly different at $P < 0.05$; n=3 per treatment.

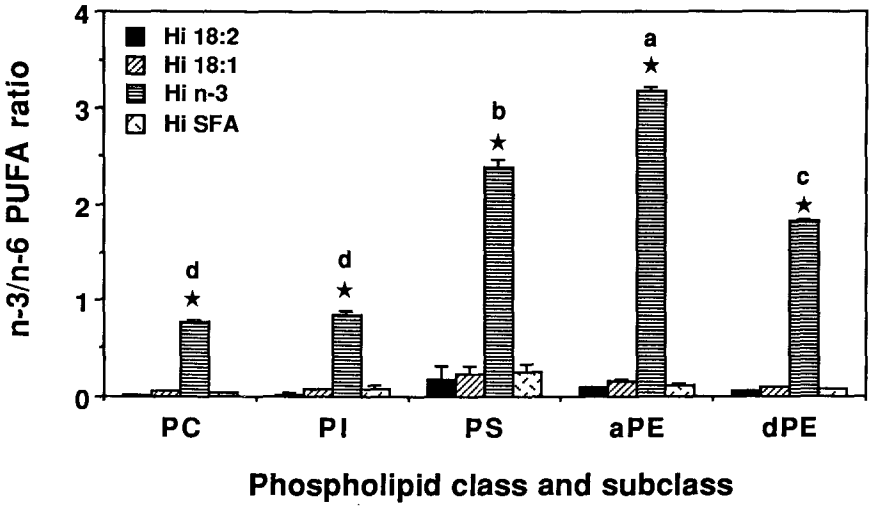

FIG. 2. Ratio of n-3 PUFA/n-6 PUFA of phospholipids. Results represent mean ± SEM (n=3). Values bearing an asterisk are significantly different from other dietary treatments in the same phospholipid. Values with the same or no superscripts are not significantly different ($P > 0.05$) between phospholipids from mice fed Hi n-3 diet.

similar results in murine macrophages (18), and rat neutrophils (16). However, it is clear from this study that the distinctive effect of each dietary fat on the fatty acid profile of certain phospholipids cannot be entirely predicted from dietary fatty acid composition. A similar phenomenon with the total phospholipids of rat splenocytes has been observed by Cleland and co-workers (24). It is not clear why incorporation of arachidonic acid in these phospholipids is resistant to dietary SFA, MUFA and linoleate feeding, but susceptible to n-3 PUFA feeding. Similar results have been observed using an *in vitro* cell culture system (25). These data suggest that incorporation of AA is a saturable process, which appears to compete with n-3 PUFA.

Dietary consumption of fish oil (Hi n-3) profoundly altered fatty acid composition of leukocyte phospholipids when compared to other fat sources. We observed that the sum of AA and EPA in each phospholipid from mice fed n-3 PUFA diet was always lower than the AA level from mice fed other diets (Tables 3-7). This finding is in accord

with the results of others (15,16,18). Interestingly, Hi n-3 feeding caused preferential incorporation of certain n-3 PUFA into specific phospholipid subclasses. We observed that DHA was the predominant n-3 PUFA in aPE and PS, whereas EPA was the predominant n-3 PUFA in PC and PI. The preferential location of DHA in PE and PS relative to other n-3 PUFA has been observed in retinal rods of other animals (26) and in rat liver (27). In addition, preferential incorporation of certain n-3 PUFA into specific phospholipid subclasses has been observed in human platelets (28), but not in murine macrophages (18) or rat neutrophils (16). This indicates that such preferential incorporation may be organ or cell type specific. It is not clear at present what the underlying mechanism involved in such a preferential incorporation process would be.

The ratio of n-3 PUFA/n-6 PUFA in mice fed the Hi n-3 diet were quite different between phospholipid classes and subclasses (Fig. 2). The highest ratio of n-3 PUFA/n-6 PUFA coincided with those phospholipids which had DHA as the predominant n-3 PUFA. This may indicate that either *in situ* formation through elongation and/or desaturation or preferential acylation of DHA is more active in aPE and PS than in PC and PI.

In the present study, we observed that dietary fat source did not alter the percentage of spleen lymphocytes, macrophages as well as polymorphonuclear leukocytes (Table 2). This suggests that our lipid analysis was done on a similar cell population across diet groups. However, we consistently observed that dietary fat source significantly influenced spleen weight, and the number of leukocytes, lymphocytes and macrophages recovered from those spleens. The enlargement of the spleen and the increase in spleen cell yield due to fish oil feeding has been observed previously (29). At present, it is not known why both Hi 18:1 and Hi n-3 feeding caused splenic hyperplasia. We believe that the effect of dietary fat on spleen weight and number of lymphocytes and macrophages may be related to leukocyte membrane phospholipid fatty acid composition. For instance, both the Hi 18:2 group and Hi SFA group were more alike in their phospholipid fatty acid profile and they both had similar spleen weight and number of lymphocytes and macrophages. The incorporation of MUFA and n-3 PUFA into cellular membrane phospholipids may cause a change in membrane integrity, membrane associated enzyme and receptor activities, lipid mediator biosynthesis and the production of cytokines. This may directly or indirectly influence the immune cell proliferation and/or migration (30).

We observed that source of dietary fat did not influence the overall phospholipid composition of spleen leukocytes. Our data are in agreement with the results reported by Careaga-Houck and Sprecher (16), who showed that corn oil and fish oil feeding had no effect on rat neutrophil phospholipid content. Similarly, Chapkin and Coble (31) have demonstrated that dietary fat source did not alter phospholipid class or subclass levels in murine kidneys. However, Chapkin and Carmichael (18) have shown that dietary fat can affect PE subclass levels in rat peritoneal macrophages. Such an effect may be organ or cell type specific.

In conclusion, the present study has shown that dietary fat source can have a profound effect on the fatty acid composition of murine leukocyte membrane phospholipids, and that such effects were unique for each phospholipid class or subclass. Hi n-3 feeding can result in preferential incorporation of n-3 PUFA, particularly DHA into aPE, PS and, to a lesser extent, dPE. Source of dietary fat can also influence spleen weight and cellularity, and such effects may be correlated to the fatty acid composition of phospholipids.

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Effect of Human Mammary MX-1 Tumor on Plasma Free Fatty Acids in Fasted and Fasted-Refed Nude Mice

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We hypothesized that tumor-bearing (TB) nude mice, because they are reported to have no detectable, circulating tumor necrosis factor (TNF)/cachectin, would regulate their plasma free fatty acid (FFA) levels normally when fasted and refed. We compared levels of individual plasma FFA in response to fasting (24 hr) and to refeeding (for 20 hr after fasting) a fat-free, 65% sucrose diet in control, nude mice and in mice bearing 1.3 ± 0.4 g MX-1 tumors. Total plasma FFA levels were typically high in 24-hr fasted mice [mean concentrations in μM ± SE (n); controls 515 ± 63 (6); TB, 625 ± 63 (6)]. FFA levels were reduced by 65% in each group in response to refeeding. Each major plasma FFA species fell in response to refeeding, except arachidonate, which did not change significantly (fasted *vs.* fasted-refed concentrations) in either controls or TB mice. In refed mice, the molar FFA ratio of oleate to linoleate rose; however, that of oleate to arachidonate fell markedly. TB nude mice had normal appetites. We conclude that all species of FFA were mobilized from adipose tissue in a normal manner in TB nude mice; therefore, regulation of adipose triacylglycerol fatty acid mobilization (as plasma FFA) by dietary sugar is probably not affected by MX-1 tumor growth in these mice. Our findings are consistent with the hypothesis that nude mice may be unable to secrete TNF/cachectin in response to tumor growth, but they do not establish whether or not endogenous, circulating TNF/cachectin increases FFA mobilization in any TB animal.
Lipids 27, 33–37 (1992).

Immunodeficient mice, such as nude BALB/c mice, are commonly used as experimental models to study factors that regulate growth of human cancers and to evaluate the effects of anti-cancer therapies against human cancers *in vivo*. Mice with defective immune systems are also potentially useful for studying the effects of cancer-derived and host-derived cytokines upon both the cancer and the host. Effects of tumor necrosis factor (TNF)/cachectin, produced by Chinese hamster ovarian (CHO) tumors transfected with the mouse TNF/cachectin gene, have been reported in nude mice (1). However, to our knowledge, the cachectic effects of human carcinomas on adipose tissue or on the regulation of plasma free fatty acid (FFA) mobilization by diet in nude mice have not been studied. Such studies would seem to be of considerable interest because nude mice bearing large CHO tumor burdens apparently do not have detectable levels of circulating TNF/cachectin (1), nor do they increase circulating TNF/cachectin levels in response to bacterial vaccines and other agents as do normal mice, despite being able to increase production of macrophages (2). This abnormality in TNF production may be attributable to the total or partial absence of T-cells which are required in order for certain agents to activate macrophages so that they can respond to substances that induce TNF/cachectin production and/or secretion by the macrophages (2,3).

Cancer cachexia, and loss of body fat in particular, may be due to the effects of cytokines such as TNF/cachectin acting on appetite centers (1) or on adipose tissue to increase triacylglycerol fatty acid (FA) mobilization (4–7) and/or to prevent deposition of very low density lipoprotein (VLDL) and chylomicron triacylglycerol fatty acids (TGFA) by inhibition of lipoprotein lipase synthesis (8–10). According to these hypotheses, one would not expect to observe cancer cachexia and increased mobilization of plasma free fatty acids (FFA) in nude mice bearing human carcinomas in the absence of significant levels of circulating TNF/cachectin.

In the present study we have examined total and individual plasma FFA concentrations following both fasting and refeeding, as indices of plasma FFA mobilization and regulation, in nude mice bearing MX-1 mammary carcinomas and in their non-tumor-bearing controls. We have also studied the effects of MX-1 tumor growth on food intake and, in a separate study on adiposity (unpublished data). Our studies are consistent with, but do not prove, the hypothesis that in the absence of detectable circulating TNF/cachectin (1) both appetite and the dietary regulation of fat metabolism in adipose tissue are unaffected by tumor growth, at least in nude mice bearing MX-1 mammary carcinomas.

METHODS

Mice. Female BALB/c (nu/nu), 7–8-weeks-old, were obtained from Simonson (Gilroy, CA). They were housed (four mice/cage) in autoclaved cages with filter tops and bedding in a temperature-controlled room with a 12-hr light (daytime) and a 12-hr dark cycle. The average weights (± SE) of the control and TB mice on the day of the experiment were as follows: fasted controls, 20.4 ± 0.22; fasted-refed controls, 22.4 ± 0.65; fasted TB (MX-1), 20.6 ± 0.23; and fasted-refed TB, 22.8 ± 1.0 g.

Tumor. The estrogen receptor-negative transplantable human breast carcinoma, MX-1, derived from a primary breast tumor, was obtained originally from the EG & G Mason Research Institute (Worchester, MA) and transplanted serially in nude mice since 1983. Under sterile conditions, the least necrotic pieces of a well-developed MX-1 tumor from a donor mouse was minced with a scalpel in

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Abbreviations: BHT, 3,5-di-*t*-butyl-4-hydroxytoluene; CHO, Chinese hamster ovarian; DME, Dulbecco's modified Eagle's (medium); EDTA, ethylenediaminetetraacetic acid; FA, fatty acids; FAME, fatty acid methyl esters; FFA, free fatty acids (unesterified fatty acids); GLC, gas-liquid chromatography; TB, tumor-bearing; TGFA, triacylglycerol fatty acids; TLC, thin-layer chromatography; TNF/cachectin, tumor necrosis factor; VLDL, very low density lipoprotein.

a minimal volume of Dulbecco's modified Eagle's (DME) cell culture medium, passed through a syringe without a needle and then through a syringe with a 16-gauge needle attached. Recipient nude mice were then injected subcutaneously, proximal to the right no. 4 mammary gland, with the MX-1 tumor suspended as a gel in DME cell culture medium. The volume of tumor gel suspension injected was 50 μL delivered through a 16-gauge needle attached to a 1-mL syringe. After each experiment, tumors were either excised and their sizes estimated based upon direct wet weight measurements or upon the tumor volumes measured *in situ* and assuming a density of 1.0 (a minimal estimate). Tumor volumes (V) were calculated from caliper measurements of 3 perpendicular dimensions; thus, V = (length × width × depth)/2. The mean tumor weight (± SE) of 12 mice was 1.27 ± 0.37 g, 5–7 weeks after tumor inoculation.

Diet. The mice were fed Purina laboratory chow (5058) *ad libitum* until they were fasted for 24 hr. Those that were refed for 20 hr following the fasting period were given fat-free pellets that contained, by weight, 64.8% sucrose, 24.0% vitamin-free casein, 3.75% salt mixture (AIN #76), 2.2% vitamin fortification mixture (ICN Nutritional Biochemicals, Cleveland, OH), 0.3% methionine, 5.0% cellulose, and 0.01% BHT (3,5-di-*t*-butyl-4-hydroxytoluene). Estimates of individual daily food intakes during the 72-hr period before fasting, and when the mice were still eating Purina laboratory chow, were based upon the average food consumed by 32 control mice and by 32 TB mice. It was from each of these populations that 24 mice were chosen (randomly, in the case of controls, and selected to obtain the most homogenous tumor sizes, for the TB group). Daily food intake per mouse was calculated using a factor of 12 (four mice/cage × three-day intake). The average food consumed during the 20-hr refeeding period (65% sucrose, fat-free diet) was based on the average food eaten by 12 control mice and by 12 TB mice. Food intakes are given in the Results section.

Experimental protocol. Twelve chow-fed animals (six control and six bearing MX-1 tumors) were fasted for 24 hr and then refed the special fat-free diet for 20 hr. The mice were sacrificed between 10 a.m. and 12 noon along with another 12 animals (six controls and six TB mice) fasted for 24 hr. Retroorbital blood or blood following decapitation, both under diethyl ether anesthesia (30 seconds before killing), was collected in tubes containing solid ethylenediaminetetraacetic acid (EDTA). The stomach contents in all fed mice were examined and found to be between 3/4 full and full.

Plasma FFA analysis. The standard reference, "Method I", of Lin *et al.* (11) was used without modification. Briefly, this method consisted of extracting total lipids (12) from 0.1 mL of plasma to which 5 μg of heptadecanoic acid had been added as internal standard, thin-layer chromatographic (TLC) separation of the FFA fraction, scraping of the FFA zone, methylation of FFA with a diazomethane ethereal solution added to the TLC scrapings, quantitative transfer into hexane of the fatty acid methyl esters (FAME), concentration, and gas-liquid chromatographic (GLC) separation of FAME using a 5 μL aliquot of the hexane solution; the instrumentation and GLC conditions were exactly as described previously (11). Similar results were obtained when FFA, separated by TLC, were methylated by either diazomethane (13) or acetyl chloride (14).

RESULTS

Food intake of MX-1 and control nude mice during the 20-hr refeeding period. Since cancer growth often induces anorexia in rodents, and anorexia influences FFA mobilization, it was important to monitor the food intake carefully both before the fasting period was begun and again during the 20-hr refeeding period. The average daily intakes of Purina laboratory chow in the period preceding the fasting period were as follows: controls, 3.5 ± 0.064 g/mouse; TB, 3.3 ± 0.073 g/mouse (mean ± SE; n = 8 cages/group of either 32 control or 32 TB mice). The difference was not statistically significant. Both groups of animals also ate nearly identical quantities of food (65% sucrose, fat-free diet) during the 20-hr refeeding period, as follows: controls, 4.2 ± 0.32 g/mouse; TB, 4.4 ± 0.21 g/mouse (mean ± SE; n = 3 cages/group of either 12 control or 12 TB mice).

Effect of fasting and refeeding on plasma FFA levels of control nude mice with and without MX-1 tumors. As shown in Table 1 the levels of plasma FFA as measured by GLC were slightly higher in TB mice than in controls, but the differences were minor and statistically insignificant. Plasma FFA levels fell in response to refeeding a high-sucrose, fat-free diet by 63% in the nude controls (from 515 to 190 μM) and, to a similar extent, 65% in the MX-1-bearing nude mice (from 625 to 220 μM). Therefore, this human mammary carcinoma had no apparent effect on this insulin-dependent regulatory process, *i.e.*, on suppression of hormone-sensitive lipase and augmentation of FFA reesterification in adipose tissue, the net effect of which is usually reflected in the plasma FFA levels (15–17).

All of the major individual C_{16} and C_{18} FFA, which constitute the bulk of the component FFA showed highly significant decreases upon refeeding, and the changes in each were essentially the same in TB and in control mice (Fig. 1). The greatest percentage changes were found for 18:2 and 18:1 (decreases of about 83% and 74%, respectively), followed by 16:1 and 16:0 (decreases of 65% and 55%, respectively). The change in 18:0 levels, about 35%, was less than that of the others, with the exception of 20:4, which was unique in that its plasma FFA concentration showed no change in response to refeeding.

The relationships of the plasma FFA oleate and FFA arachidonate concentrations to the mean concentrations

TABLE 1

Effect of Fasting (24 hr) and Fasting Followed by Refeeding a Fat-Free 65% Sucrose Diet for 20 hr on Plasma FFA Levels of MX-1 Tumor-Bearing and Control Nude Mice[a]

Mice	Plasma FFA concentration [x ± SE (n)]	Decrease (%)
Controls		
Fasted	515 ± 63 (6)	—
Fasted-refed	190 ± 17 (6)	63
MX-1 Tumor-bearing		
Fasted	625 ± 63 (6)	—
Fasted-refed	220 ± 9.6 (6)	65

[a]Units (μmol/L) based on the six major GLC peaks in relation to a 17:0 FAME standard. As an approximation, FFA mol. wt. was assumed to be 270 for each individual FFA. Statistical evaluation: Fasted *vs.* fasted-refed, $p \leq 0.001$, both controls and TB groups.

of each of the other FFA moieties, in the fasted and fasted-refed states for both control and TB mice, are summarized in Figure 2. First, oleic acid (18:1) is shown as the reference FFA (Fig. 2, upper), selected because it was the most highly concentrated individual FFA in the fasted state in both the control and the TB mice (Fig. 1). The ratio of 18:1 to every other FFA was greater than 1 and greater in the fasted state than in the fasted-refed state in every case except 18:1/18:2. In the fasted state, the relative approximate molar ratios of 18:1 to each of the other FFA moieties were similar to those in the fasted-refed state; however, there were some noticeable differences. First, the ratio of 18:1 to 18:2 rose from 2 to 3 in fasted-refed mice, the only instance in which the ratio did not fall upon refeeding. For example, the ratio of 18:1 to 18:0 fell from 3 to 1. Second, the ratio of 18:1 to 20:4 showed a striking difference between the two nutritional states in both TB and control mice. The ratio averaged 19 (controls) and 13 (TB) in the fasted state and fell to about 4 in both groups when they were refed. Note that these are the ratios of the mean values and not the means of the individual ratios, which were highly variable, especially with respect to levels of 20:4. The difference in this ratio between control and TB mice in the fasted state (*i.e.*, 19 *vs.* 13) was not significant.

The second relationship explored was the mean molar ratio of arachidonate (20:4) to each major FFA (Fig. 2). Arachidonate was chosen here as the reference FFA because, along with linoleate, it is thought to be of special significance with respect to the regulation of tumor growth (18,19), and it is the one plasma FFA whose concentration remained unchanged after refeeding. As shown in the bottom half of Figure 2 the ratios of 20:4 to each of the other FFA increased upon refeeding, with the most striking increases occurring in relation to 18:2 (ratio rising from 0.1 to 0.75) and to 16:1 (ratio rising from 0.5 to 1.5, average). Note that 20:4 is less concentrated than all other plasma FFA (ratio <1.0), except 16:1, and is only more concentrated than 16:1 in the fasted-refed state.

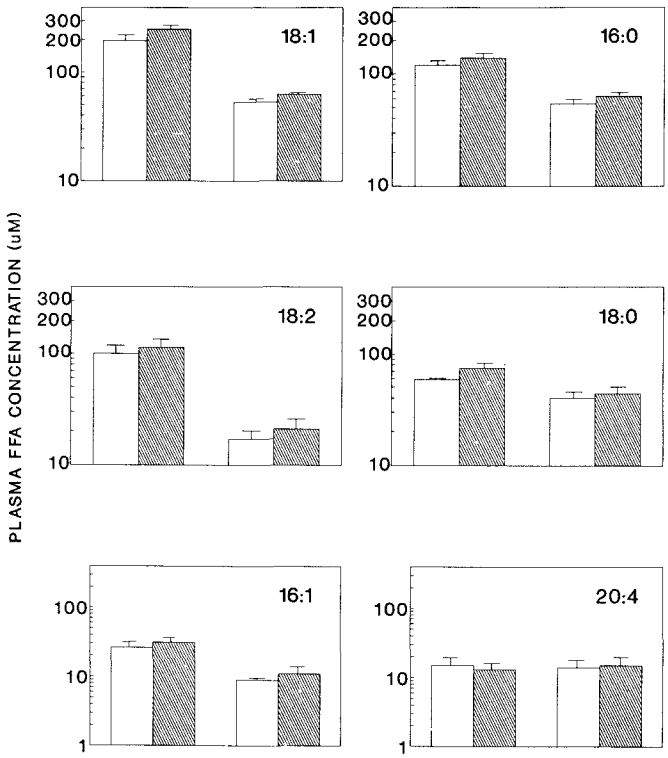

FIG. 1. Effect of fasting and fasting-refeeding on the mean concentrations of individual plasma FFA in control nude mice and in MX-1 tumor-bearing mice. The Figure illustrates that the average response to refeeding of each individual FFA is the same in the TB as well as in the control groups and that each of the major individual FFA species is significantly affected by refeeding, with the notable exception of arachidonate (20:4). Each bar represents the mean values (vertical bar, ± SE) of six mice for each of four groups: two bars on the left of each frame are mean values for the mice that were fasted for 24 hr; the two bars on the right of each frame are mean values for the 24-hr fasted, 20-hr refed mice. Open bars, control nude mice; hatched bars, TB mice. Note the use of a logarithmic scale for plasma FFA concentrations and the different scale range for 16:1 and 20:4 compared to the other four FFA. Statistical analysis, fasted *vs.* refed, for each FFA species (except 20:4, n.s.) in both control and TB groups was $p \leq 0.001$, except for the following, each of which was statistically significant at $p \leq 0.01$—18:0 (both controls and TB) and 16:1 (TB).

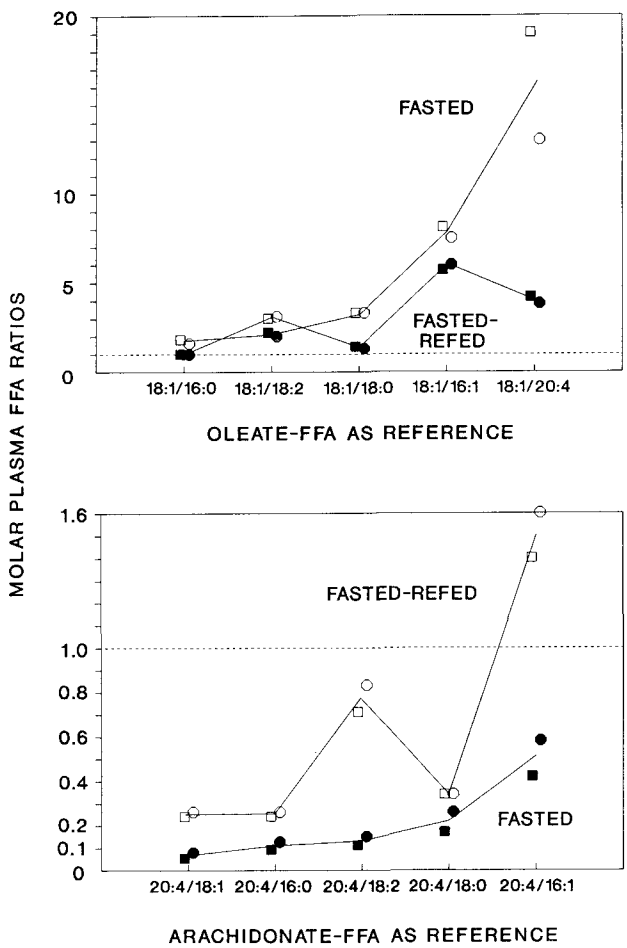

FIG. 2. Relative molar ratios of individual plasma FFA in fasted and fasted-refed nude control and MX-1 tumor-bearing mice. In the upper Figure, the mean molar ratios are shown for oleate (18:1), the most abundant FFA in the fasted state, to each of the other five major FFA species. In the lower Figure, the mean molar ratios of the concentration of arachidonate (20:4) in relation the other five major FFA are shown. A reference line (at 1.0), corresponding to an equimolar ratio, is indicated in each Figure. The patterns for control and tumor-bearing mice were nearly identical. Symbols: controls, fasted (○), refed (●); TB, fasted (□), and refed (■).

DISCUSSION

Although immunodeficient, nude mice are widely used to study such important aspects of human carcinoma growth as its detection, prevention and treatment, little information is available regarding fat metabolism in these mice. Such studies are highly relevant because of the growth-promoting effects of certain dietary fats, the possibility that linoleate and arachidonate transport to tumors may be rate-limiting for tumor-growth, and the frequent association of increased mobilization of adipose tissue fat stores and of hypertriglyceridemia with cancer in both rodent models and human subjects. Nude mice are also of potentially special significance because they lack T-cells, which control the production of TNF/cachectin by macrophages (2,3). Since this cytokine has been implicated in cancer cachexia and in abnormalities of fat metabolism in TB mice, we have hypothesized that, in the assumed absence of TNF/cachectin, fat metabolism will be unaltered in nude mice bearing a human mammary carcinoma (MX-1).

In the present study, the first of several designed to provide information relevant to this hypothesis, we found that nude mice bearing MX-1 human mammary carcinomas did not have elevated levels of any plasma FFA in either the fasted or fed state. Therefore, it is unlikely that tumor growth increases FFA mobilization in these mice. Furthermore, since plasma FFA levels are determined by insulin-dependent processes, *i.e.*, hormone-sensitive lipase activity and FFA reesterification in adipose tissue (15–17) in relation to plasma FFA utilization rates, no tumor-associated insulin resistance or deficiency was apparent in our TB mice.

Although several authors have suggested that FFA mobilization is increased in TB rodents (*e.g.*, refs. 4–6), mice bearing Ehrlich ascites tumors respond normally to fasting and refeeding by raising and lowering plasma FFA levels and production rates (17), and briefly fasted lymphoma-bearing mice produce FFA at a somewhat slower than normal rate (20,21). At night (simulated), palmitate is mobilized and oxidized at an increased rate in lymphoma-bearing mice (21); however, later studies have shown that this abnormality results from anorexia rather than from defective regulatory mechanisms (Kannan, R., Gan-Elepano, M., and Baker, N., unpublished observations). Our nude mice with MX-1 tumors were not anorexic at the tumor burden studied; therefore, elevated plasma FFA mobilization rates secondary to decreased caloric intake would not be anticipated.

Elevated nocturnal mobilization of plasma FFA in anorexic lymphoma-bearing mice (21) is associated with marked loss of body fat. We have found that the mean inguinal fat pad weights of MX-1 TB mice and their nude controls do not differ significantly (Baker, N., Lin, C., Ceriani, R.L., Blank, E.W., Chengson, R.J., and Corten, C.E., unpublished observations). Apparently, the MX-1 tumor, at the present tumor burden, does not cause a net loss of body fat, a finding consistent with the normal FFA levels and responses to fasting and feeding.

We assume that nude mice bearing human mammary MX-1 tumors, like those bearing CHO tumors (1), cannot produce detectable levels of circulating TNF/cachectin because they lack T cells (2,3). This may account for their normal appetites, their apparent ability to conserve body fat, and their normal regulation of FFA mobilization from adipose TGFA. However, it is still conjectural as to whether or not endogenously generated TNF/cachectin plays any significant role with respect to FFA mobilization in TB mice or in cancer patients (22). Even the hypothesis that the lipoprotein lipase activity of adipose tissue from human cancer subjects is inhibited by TNF/cachectin has been questioned recently (23).

Defective regulation of FFA mobilization and oxidation after ingestion of multiple, small glucose test meals has been reported in humans with cancer (24). When mice are given multiple small glucose-rich test meals, each successive test meal greatly increases the efficiency with which the glucose in the next test meal is cleared from the circulation, provided that there is protein and/or other (non-fat) additives included in the complete diet (25). Whether human subjects with cancer can regulate fatty acid mobilization in a normal fashion after eating regular meals remains to be determined.

Finally, we call attention to the unique behavior of arachidonate in response to fasting and refeeding, in both control and TB mice. Apparently there is a special homeostatic mechanism that maintains the plasma FFA level of this important substrate for the cyclooxygenase and lipoxygenase pathways at a constant level under conditions in which all of the other major FFA are undergoing significant and typically large changes in concentration. Additional experiments are needed to confirm this observation in other strains of mice, as well as in other species, and to determine both the regulatory processes involved and the potential physiological significance of the phenomenon. It is generally considered that membrane phospholipids and diglycerides provide the substrate for the cyclooxygenase and lipoxygenase pathways in tissues. It would seem important to determine the extent to which plasma FFA arachidonate complexed to albumin, serves as a precursor for these pathways in various host tissues, especially in platelets, endothelial cells, and cells of the immune system, as well as in the tumor cells themselves, in each of the dietary states studied here.

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Comparative Uptake of α- and γ-Tocopherol by Human Endothelial Cells

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The intake of γ-tocopherol by North Americans is generally higher than that of α-tocopherol. However, the levels of α-tocopherol in human blood have consistently been shown to be higher than those of γ-tocopherol suggesting differential cellular retention of the two tocopherol forms. We sought to resolve this question by studying tocopherol metabolism by human endothelial cells in culture. The time- and dose-dependent uptake of γ-tocopherol by endothelial cells was similar to that of α-tocopherol. To determine the comparative uptake between α- and γ-tocopherol, we adopted two approaches in which cells were enriched with either increasing concentrations of an equimolar mixture of α- and γ-tocopherol; or cells were enriched with a fixed concentration of tocopherols in which the α to γ ratio was varied. Our results indicated that there was a preferential uptake of γ-tocopherol by the cells. When cells were enriched with either α- or γ-tocopherol and the disappearance of individual tocopherols was monitored over time, γ-tocopherol exhibited a faster rate of disappearance. The faster turnover of γ-tocopherol can explain the discrepancy between high intake and low retention of γ-tocopherol in man.
***Lipids* 27, 38–41 (1992).**

γ-Tocopherol represents a significant portion of total tocopherol in the North American diet. Depending on the type of dietary fat used, the daily intake of γ-tocopherol can easily be twice that of the biologically more active α-form (1,2). Despite this disproportionally higher intake of γ-tocopherol, analyses by different laboratories of human plasma and blood cells consistently revealed a high alpha to gamma ratio ranging from 3.4 to 6.5 (3–5). This may suggest that the turnover rates of the two tocopherols are different and that the retention of γ-tocopherol is lower than that of the α-form.

In vivo, the tocopherols are transported by lipoproteins and the amounts present in the circulation are dependent upon a complex interplay of various physiological factors including dietary intake, absorption, peripheral clearance, hepatic secretion, and clearance of lipoprotein remnants (6–8). The biological activity of γ-tocopherol is generally thought to be lower than that of α-tocopherol, and depending on the type of bioassays used to assess bioactivity, γ-tocopherol usually possesses only 10–20% of the activity of the α-form (9,10). However, when assessed *in vitro*, γ-tocopherol has been shown to be a better antioxidant than the α-form (11,12). Thus, the biological activities of the tocopherols do not correlate well with their apparent antioxidant property.

We have recently developed a method to disperse *RRR*-α-tocopherol in culture medium, and we have used this approach to study the uptake and disappearance of α-tocopherol by endothelial cells isolated from human umbilical cord veins (13). The incorporation of α-tocopherol by these cells allows complete control of cellular tocopherol levels. This endothelial cell model thus permitted us to study the effects of tocopherol on various biochemical processes in these cells (14,15). The present paper describes the uptake and disappearance of γ-tocopherol as well as the comparative uptake of γ- and α-tocopherol by human endothelial cells.

MATERIALS AND METHODS

Materials. Medium 199 with Hank's salt and L-glutamine, heat-inactivated fetal bovine serum, sodium penicillin G (10,000 units/mL), streptomycin sulfate (10,000 μg/mL), fungizone, trypsin-EDTA, and culture flasks and dishes were purchased from Gibco (Burlington, Ontario, Canada). *All-rac*-α-tocopheryl acetate, HEPES, *i.e.* 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid, collagenase type IV, gentamycin sulfate, and heparin (180 units/mg) were from Sigma (St. Louis, MO). Endothelial cell growth supplement was from Collaborative Research (Bedford, MA). γ-Tocopherol was a generous gift from Eisai Co. Ltd. (Tokyo, Japan). High-performance liquid chromatography (HPLC) grade solvents were from BDH Chemicals Inc. (Toronto, Ontario, Canada).

Endothelial cell cultures. Endothelial cells isolated from human umbilical veins were cultured in medium 199 as previously described (13). Medium 199, pH 7.4, was supplemented with heat-inactivated fetal bovine serum (10%), endothelial cell growth supplement (30 μg/mL), heparin (90 μg/mL), HEPES (25 mM), gentamicin sulfate (40 μg/mL), penicillin (100 units/mL), streptomycin sulfate (100 μg/mL) and fungizone (amphotericin B 2.5 μg/mL). Cells were subcultured after confluent monolayers were detached with trypsin-ethylenediaminetetraacetic acid disodium dihydrate (EDTA) (0.05%) and divided at a 1:2 split ratio. Cells from passages 1–3 were used in all experiments.

Enrichment of γ-tocopherol in culture medium. γ-Tocopherol was enriched in the culture medium in the same manner as we previously enriched α-tocopherol (13–15). Briefly, γ-tocopherol dissolved in dimethyl sulfoxide (DMSO) was added to heat-inactivated fetal calf serum (10% of medium volume). The tocopherol and the serum mixture were vortexed vigorously and incubated in the dark at 37°C for 15 min. Medium 199 and other culture reagents were then added, and the culture medium was incubated at 37°C for another 15 min, before the medium was added to the endothelial cells. The control medium contained the same amount of DMSO which did not exceed 0.1% of total culture medium.

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid disodium dihydrate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HPLC, high-performance liquid chromatography; UV, ultraviolet.

Lipid extraction and determination of tocopherol and cell numbers. The cells were harvested by trypsinization and were washed twice with HEPES buffered saline (HBS) containing 0.1% essential fatty acid-free bovine serum albumin. The cells were resuspended in HBS and an aliquot was taken for cell counting with a hemocytometer. Total cellular lipids were extracted by the method of Bligh and Dyer (16), in the presence of *all-rac*-α-tocopheryl acetate as internal standard. Cellular tocopherols were separated by reversed phase HPLC using ultraviolet (UV) detection (280 nm). The mobile phase contained methanol/water/trifluoroacetic acid (99:1:0.1, v/v/v). The flow rate through a Bondpak C-18 column was adjusted to 1.5 mL/min. The tocopherols were quantitated by peak/height ratio relative to the internal standard (tocopheryl acetate) as described (13). Tocopherol values were expressed in nmol/10^6 cells.

RESULTS

To define the time course of γ-tocopherol incorporation, confluent endothelial cell monolayers were incubated with 23 μM γ-tocopherol for various time periods. The monolayers were then rinsed with buffer containing bovine serum albumin (BSA) to remove non-specifically bound γ-tocopherol from the cell surface, and cellular tocopherol was measured after total lipid extraction. Figure 1 shows that the uptake of cellular γ-tocopherol increased proportionally with incubation time and that after 8 hr maximum incorporation of γ-tocopherol was attained. The dose dependence of γ-tocopherol incorporation was followed by incubating confluent monolayers in medium enriched with increasing concentrations of γ-tocopherol (12–92 μM). After 4 hr, uptake of γ-tocopherol by endothelial cells was directly proportional to the amount of γ-tocopherol supplemented to the medium (Fig. 2). This time- and dose-dependence of γ-tocopherol uptake is strikingly similar to that previously observed for α-tocopherol (13).

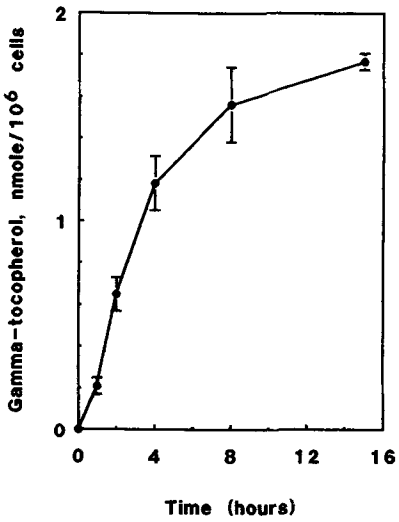

FIG. 1. Time course of γ-tocopherol incorporation by human endothelial cells. Confluent monolayers were incubated with 23 μM of γ-tocopherol; at indicated time points, cells were harvested and total lipids were extracted. Tocopherol was immediately determined by HPLC as described in Materials and Methods. Values are means ± SD of three dishes.

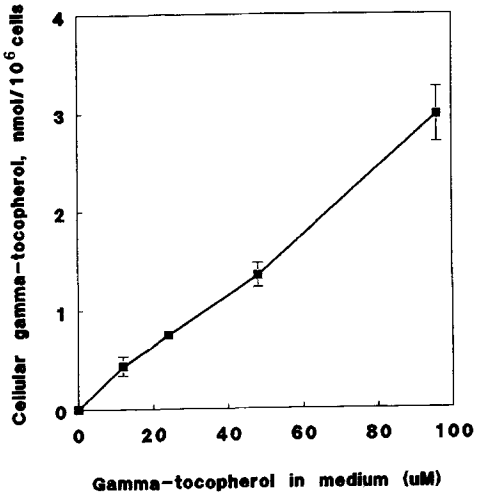

FIG. 2. Dose-dependent uptake of γ-tocopherol by human endothelial cells. Confluent monolayers were incubated with indicated γ-tocopherol concentrations for 4 hr at which time cells were harvested and total lipids were extracted. Tocopherol was determined by HPLC as described in Materials and Methods. Values are means ± SD of three dishes. Absence of deviation bars in data point denotes bars are within points.

To compare cellular uptake of α- and γ-tocopherol, we adopted two approaches in which cells were either enriched at increasing concentrations of an equimolar mixture of α- and γ-tocopherol, or the cells were enriched at a given total tocopherol concentration (46 μM) with the α to γ ratio being varied from 0.14 to 5.0. Figure 3 shows that after 4 hr of incubation with medium containing an equimolar mixture of α- and γ-tocopherol, net cellular uptake of γ-tocopherol was consistently higher than that of α-tocopherol at all concentrations tested. These data indicated that when α- and γ-tocopherol were present at equimolar concentration in the culture medium, there was a preferential uptake of γ-tocopherol. To follow up on this observation, cells were incubated for 4 hr with a 46 μM

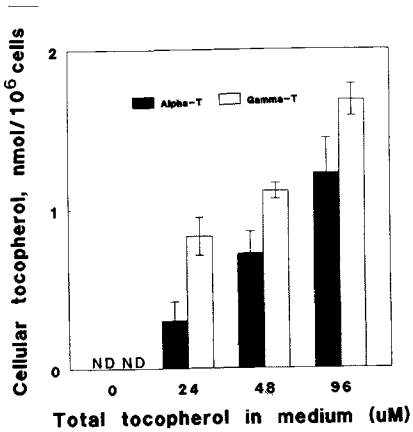

FIG. 3. Competitive uptake of α- and γ-tocopherols by human endothelial cells. Confluent monolayers were incubated with equimolar amounts of α- and γ-tocopherol. Total concentrations of α- plus γ-tocopherols are indicated on the x-axis. After 4 hr, cellular tocopherols were determined as described in Materials and Methods. Values are means ± SD of 3 dishes; N.D., not detectable.

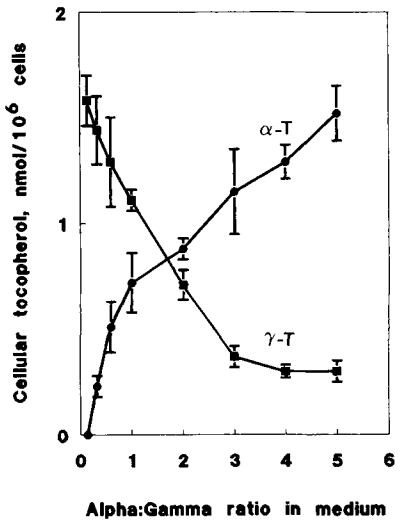

FIG. 4. Competitive uptake of α- and γ-tocopherols by human endothelial cells. Confluent monolayers were incubated for 4 hr with mixtures of α- and γ-tocopherols at the indicated α/γ ratios. Total concentration of α- and γ-tocopherol in the mixture was 46 μM. Cellular lipids were extracted and tocopherols were determined as described in Materials and Methods. Values are means ± SD of three dishes.

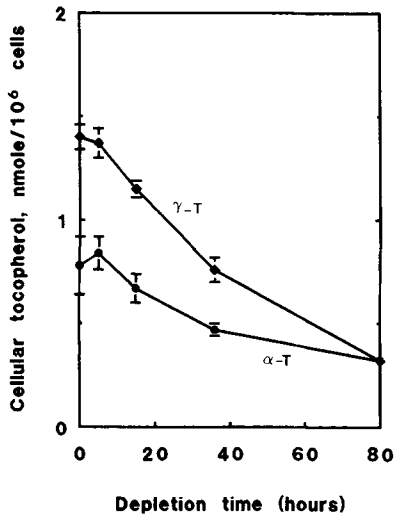

FIG. 5. Disappearance of α- and γ-tocopherol from human endothelial cells. Confluent monolayers were incubated with 23 μM of either α- or γ-tocopherol for 4 hr, after which the tocopherol-enriched media were removed. The cells were rinsed with buffer containing 0.25% BSA and reincubated with fresh culture medium without added tocopherol. At indicated time points, cells were harvested, total lipids were extracted, and tocopherol was determined as described in Materials and Methods. Values are means ± SD of three dishes.

mixture of α- and γ-tocopherol in which the ratio of α/γ was varied from 0.14 to 5.0, and incorporation of α- and γ-tocopherol into cells was measured. Figure 4 shows that the two tocopherols generally displaced each other as the α/γ ratio in the medium was varied. When the α/γ ratio was equal to 1, cellular uptake of γ-tocopherol was higher than that of α-tocopherol, and at an α/γ ratio of 2, the cells retained virtually the same amount of α- and γ-tocopherol. Thus, the preferential uptake of γ-tocopherol is clearly evident.

TABLE 1

Half-Life of α- and γ-Tocopherol Retained in Cultured Human Endothelial Cells[a]

Half-life (hr)	
α-Tocopherol[b]	γ-Tocopherol
65 ± 9	52.5 ± 5

[a] Endothelial monolayers were incubated with 23.2 μM of either α- or γ-tocopherol for 4 hr at which time the tocopherol-enriched medium was removed and replaced with fresh medium without tocopherol. Cellular tocopherol was monitored over time as described in Figure 5. The half-life was calculated from linear extrapolation of the plots as previously described (13) and values were means ± SD for 3 separate experiments.

[b] Cells were incubated with 20% fetal calf serum. The amount of tocopherol incorporated was not different at 10 or 20% fetal calf serum. Results from ref. (13).

To compare the individual rates of uptake and disappearance of α- and γ-tocopherol, cells were incubated with either α- or γ-tocopherol (23 μM) for 4 hr, and the disappearance of individual tocopherols was monitored after the culture medium had been replaced with fresh medium that was not tocopherol-supplemented. Figure 5 shows that when equal concentrations of the two tocopherols were incubated, cellular uptake of γ-tocopherol was higher than that of α-tocopherol. However, the rate of γ-tocopherol disappearance from the cells was more rapid. After 80 hr of depletion, cellular retention of the two tocopherols was similar. When the half-lives of α- and γ-tocopherol were calculated from the linear extrapolations of the disappearance curves, γ-tocopherol showed a faster rate of disappearance than did α-tocopherol. Table 1 summarizes the cumulative half-lives of α- and γ-tocopherol from several experiments. The average half-life of γ-tocopherol was 12.5 hr less than that of α-tocopherol, indicating that although the uptake of γ-tocopherol was rapid, the rate of its disappearance from the cells was also faster than that of α-tocopherol.

DISCUSSION

In a previous study, we reported the development of a human endothelial cell culture system suitable for the study of α-tocopherol uptake and disappearance (13). The usefulness of this system is enhanced by the very low amount of tocopherol present in endothelial cells isolated from human umbilical cord vein and because tocopherol becomes virtually undetectable after 2-3 cell passages. This permits studies over a wide range of tocopherol concentrations upon solubilization of the tocopherol in the culture medium aided by DMSO (13).

We sought to define the competition between α- and γ-tocopherol by using this human cell model. Figures 1 and 2 show that the uptake of γ-tocopherol as function of time and dose closely resembles that of the α-form as previously described (13). However, when examined by itself, or in different combinations with α-tocopherol, the rates of γ-tocopherol uptake (Fig. 3-4) and disappearance (Fig. 5 and Table 1) are more rapid than those of the α-form.

The mechanisms underlying the faster uptake and disappearance of γ-tocopherol are not clear at present. The

absence of a 5-methyl group on the chromanol ring of γ-tocopherol (7,8-dimethyl tocol) may render it more soluble than α-tocopherol (5,7,8-trimethyl tocol) in an aqueous medium. Conversely, the presence of a 5-methyl group in α-tocopherol may facilitate incorporation of the α-form into the membrane bilayer.

In a human study, Traber and Kayden (17) have recently demonstrated that although γ-tocopherol was absorbed as well as α-tocopherol, the plasma level of γ-tocopherol declined sharply during the post-absorptive state, indicating a faster tissue clearance of γ-tocopherol. The preferential uptake of γ-tocopherol observed in the present study may be relevant to this observation. The more rapid loss of γ-tocopherol which we observed on our present human cell model is therefore consistent with retention studies described for rat (18) and man (17). The faster turnover of γ-tocopherol may help in explaining the imbalance between high intake but low tissue retention of γ-tocopherol in humans on a typical North American diet (1–5).

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Glutathione and Antioxidants Protect Microsomes Against Lipid Peroxidation and Enzyme Inactivation

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The study investigated the relationship between lipid peroxidation and enzyme inactivation in rat hepatic microsomes and whether prior inactivation of aldehyde dehydrogenase (ALDH) exacerbated inactivation of other enzymes. In microsomes incubated with 2.5 μM iron as ferric sulfate and 50 μM ascorbate, ALDH, glucose-6-phosphatase (G6Pase) and cytochrome P450 (Cyt-P450) levels decreased rapidly and concurrently with increased levels of thiobarbituric acid-reactive substances. Microsomal glutathione *S*-transferase and nicotinamide adenine dinucleotide phosphate-cytochrome *c* reductase were little affected during 1 hr of incubation. Addition of reduced glutathione partially protected and *N,N'*-diphenyl-*p*-phenylenediamine and butylated hydroxytoluene completely protected microsomes against inactivation of ALDH, G6Pase and Cyt-P450, as well as lipid peroxidation induced by iron and ascorbate. ALDH was more susceptible than G6Pase to inactivation by iron and ascorbate, and was thus an excellent marker for oxidative stress. Inhibition of ALDH by cyanamide injection of rats exacerbated the inactivation of G6Pase in microsomes incubated with 0.1 mM, but not 25 μM 4-hydroxynonenal (4-HN). 4-HN did not stimulate lipid peroxidation. Thus, 4-HN may play a minor role in microsomal enzyme inactivation. In contrast, lipid peroxyl radicals play an important role in microsomal enzyme inactivation, as evidenced by the prevention of both lipid peroxidation and enzyme inactivation by chain-breaking antioxidants.
Lipids 27, 42-45 (1992).

One result of oxidative stress is damage to membrane lipids, a process known as lipid peroxidation. Much less studied is the damage to proteins by oxidants. Possible results of protein damage include cross-linking, scission and chemical changes in individual amino acids (1). Recent studies indicate that oxidatively-damaged proteins undergo rapid proteolytic degradation, a process that has been proposed as part of the cellular secondary antioxidant defenses (2).

During oxidative stress, various reactive species, including oxygen radicals and lipid hydroperoxides, and secondary products such as aldehydes are produced. An unresolved question is whether damage to biological molecules other than membrane lipids results mainly from attack by oxygen radicals or by lipid peroxidation products (3-5). The site-specific nature of radicals and the very low steady-state concentration of hydroperoxides in living cells imply that oxygen radicals and hydroperoxides may not play a dominant role in oxidative damage (3). 4-Hydroxyalkenals such as 4-hydroxy-2,3-*trans*-nonenal (4-HN) react readily with sulfhydryl (SH) groups and are highly cytotoxic (4,6,7). Because of its relatively high concentration, diffusibility and known toxicity, 4-HN has been hypothesized as a long-sought second messenger of oxidative damage (3,4,6). However, this hypothesis has been disputed (5).

4-HN was recently suggested to impair the glutathione dependent vitamin E radical reductase, thereby stimulating microsomal lipid peroxidation (8). As aldehyde dehydrogenases (ALDH) are the major aldehyde-removing enzymes in liver (9,10), inhibition of ALDH may accentuate cytotoxic effects of reactive aldehydes generated during oxidative stress. Indeed, ALDH have been suggested as protective enzymes (11-13). In the present study we investigated the relationship between membrane peroxidation and enzyme inactivation in microsomes and tested the hypothesis that tissues devoid of ALDH activity are more susceptible to *in vitro* lipid peroxidation and to inactivation of other sensitive enzymes.

MATERIALS AND METHODS

Preparation of microsomes. Liver from rats (350-400 g) fed a standard diet were homogenized in 0.9% NaCl (20%, w/v) and centrifuged at 12,000 × *g* for 10 min at 4°C. The postmitochondrial supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C. The pellet was washed in saline and centrifuged at 100,000 × *g* for 50 min at 4°C. The washed microsomal pellet was suspended in 1 mL of saline/g liver by three 20-second bursts of sonication in ice. Sonication with care to suspend the microsomal pellet is more satisfactory than homogenization and use of a detergent, as homogenization often results in some loss of samples, while use of detergent can lead to denaturation of enzymes. The average protein content as measured using the Bio-Rad method was approximately 12 mg/mL of microsomal suspension.

Incubation of microsomes with iron and ascorbate. A portion (0.5 mL) of hepatic microsomes was transferred to a serum vial that contained 3.5 mL of 0.15 M KCl/0.01 M Tris buffer (pH 7.4) such that the average protein content was approximately 1.50 mg/mL. The microsomes were incubated at 37°C with or without 1.0 mM GSH and either 0.36 mM butylated hydroxytoluene (BHT) dissolved in ethanol or 20 μM *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) dissolved in dimethyl sulfoxide. The concentrations of ethanol (0.2%) and dimethyl sulfoxide (0.1%) used did not affect lipid peroxidation. The reaction was initiated by adding ascorbic acid and ferric sulfate to final concentrations of 50 and 2.5 μM, respectively. The reaction was terminated by adding EDTA and BHT to final concentrations of 1.0 and 0.45 mM, respectively. The mixture was used immediately for lipid peroxidation measurement.

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Abbreviations: Aa, ascorbic acid; ALDH, aldehyde dehydrogenase(s); BHT, butylated hydroxytoluene; Cyt-*c*-Rx, cytochrome *c* reductase; Cyt-P450, cytochrome P450; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; EDTA, disodium ethylenedinitrilotetraacetate; GSH, reduced glutathione; G6Pase, glucose-6-phosphatase; GSH *S*-Tr, glutathione *S*-transferase; 4-HN, 4-hydroxynonenal; TBARS, thiobarbituric acid-reactive substances.

For enzyme measurement, the mixture was stored in ice for up to three days, during which there were no appreciable changes (± 5%) in lipid peroxidation and enzyme activities.

Injection of cyanamide and incubation of microsomes with 4-HN. Rats (250–300 g) fed a standard diet were injected *i.p.* with cyanamide (2.5 mg/0.1 mL/100 g body wt) or an equivalent volume of saline 30 min before they were killed. 4-HN (kindly provided by Dr. H. Esterbauer) was dried under nitrogen and dissolved in deionized water. Hepatic microsomes (approximately 12 mg protein/mL) from control or cyanamide-injected rats were prepared as described above. An aliquot (0.5 mL) of hepatic microsomes was added to 3.5 mL of the KCl/Tris buffer containing 0.25 mM NAD^+ in a serum vial. Incubation was carried out at 37°C for 1 hr with or without 4-HN. Reaction was slowed by storing the microsomes in ice and by adding BHT (final concentration, 0.45 mM).

Measurement of lipid peroxidation and enzyme activities. Thiobarbituric acid-reactive substance (TBARS) in microsomes were measured as described previously (14,15). Other measurements included ALDH (12), glucose-6-phosphatase (G6Pase) (16), NADPH cytochrome *c* reductase (Cyt-*c*-Rx) (17), cytochrome P450 (Cyt-P450) (18) and microsomal GSH *S*-transferase (GSH S-Tr) (19).

Statistical analysis. Data were subjected to either Student's *t*-test or analysis of variance, and mean values were compared using Duncan's Multiple Range test with an SAS program (SAS Institute, Cary, NC); $p < 0.05$ was considered significant.

RESULTS

Effect of GSH, BHT, and DPPD on lipid peroxidation and enzyme activities in microsomes incubated with iron and ascorbate. The time-course study showed that iron and ascorbate rapidly and strongly induced TBARS and decreased Cyt-P450 and the activity of ALDH and G6Pase (Fig. 1). However, iron and ascorbate only slightly decreased Cyt-*c*-Rx activity (Fig. 1) and did not affect microsomal GSH S-Tr activity (Table 1).

Inclusion of 1.0 mM GSH effectively decreased TBARS induced by iron and ascorbate (Fig. 1). GSH prevented the inactivation of ALDH and G6Pase and the loss of Cyt-P450 by 75, 49 and 40%, respectively, at the end of 1 hr incubation. GSH also slightly decreased the loss of Cyt-*c*-Rx activity. An increase of GSH to 2 mM did not result in further changes in TBARS and enzyme activities.

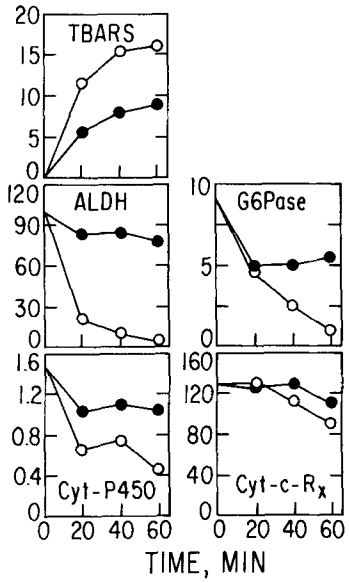

FIG. 1. Lipid peroxidation and enzyme activities in rat liver microsomes incubated with 2.5 μM ferric sulfate and 50 μM ascorbic acid at 37°C. ○, Control; and ●, 1.0 mM GSH added. Each data point is the mean of 2–4 replications of liver pooled from 3 rats. Units are (in min^{-1} mg $protein^{-1}$); TBARS, nmol; Cyt-P450, nmol; ALDH, nmol NAD^+ reduced; and Cyt-c-Rx, nmol cytochrome *c* reduced. G6Pase is μM Pi/20 min/mg protein.

Inclusion of 0.36 mM BHT or 20 μM DPPD completely inhibited lipid peroxidation induced by iron and ascorbate (Table 1). These two nonthiol antioxidants also prevented iron and ascorbate-induced loss of Cyt-P450 and of ALDH and G6Pase activities.

Effect of cyanamide injection on microsomes incubated with 4-HN. The microsomal preparations exhibited detectable activity for oxidation of 4-HN. With 0.1 mM 4-HN as substrate the activity was 10.7 ± 0.7 nmol NADH

TABLE 1

Effect of Butylated Hydroxytoluene and *N,N'*-Diphenyl-phenylenediamine on TBARS and Enzyme Activities in Hepatic Microsomes Incubated with Iron and Ascorbic Acid[a]

	Control	Fe + Aa	Fe + Aa + BHT	Fe + Aa + DPPD
ALDH	83.9 ± 3.3[b,a]	10.1 ± 2.5[b]	81.4 ± 6.2[a]	78.7 ± 1.7[a]
TBARS	0.61 ± 0.06[a]	14.0 ± 1.05[b]	0.52 ± 0.03[a]	0.45 ± 0.04[a]
Cyt-P450	1.16 ± 0.15[a]	0.60 ± 0.06[b]	1.25 ± 0.12[a]	1.28 ± 0.14[a]
GSH S-Tr	68.2 ± 5.2	62.6 ± 2.8	66.3 ± 4.5	73.2 ± 4.3
G6Pase	7.69 ± 0.44[a]	2.55 ± 0.57[b]	6.73 ± 0.77[a]	7.58 ± 0.28[a]

[a]Microsomes (1.5 mg protein/mL) were incubated at 37°C for 60 min with or without 2.5 μM ferric sulfate and 50 μM ascorbic acid (Aa). The concentrations of butylated hydroxytoluene (BHT) and *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) were 0.36 mM and 20 μM, respectively.

[b]Units are (min^{-1} mg $protein^{-1}$): ALDH, nmol NAD^+ reduced; TBARS, nmol; Cyt-P450, nmol; and GSH S-Tr, nmol CDNB conjugated. G6Pase is μmol Pi/20 min/mg protein. Data were analyzed using ANOVA and means were compared using Duncan's Multiple Range test.

Values not sharing a common superscript are significantly different ($p < 0.05$, N = 4 rats).

TABLE 2

Effect of 4-Hydroxy-2-nonenal (4-HN) on TBARS, Cytochrome P450 and Enzyme Activities in Hepatic Microsomes from Control or Cyanamide-Injected Rats[a]

	Control		Cyanamide	
	−4-HN	+4-HN	−4-HN	+4-HN
TBARS	1.01 ± 0.04[b]	1.03 ± 0.05	0.83 ± 0.17	0.81 ± 0.10
ALDH	56.9 ± 5.8[a]	37.2 ± 3.1[b]	nd[c]	nd
G6Pase	6.26 ± 0.74[a]	4.55 ± 0.70[a]	5.58 ± 0.62[a]	2.13 ± 0.28[b]
Cyt-*c*-Rx	44.5 ± 4.3	43.1 ± 4.2	47.2 ± 0.7	42.4 ± 0.7
GSH S-Tr	41.5 ± 1.6	41.8 ± 2.9	39.8 ± 1.7	31.8 ± 5.4
Cyt-P450	1.08 ± 0.07	1.00 ± 0.10	1.09 ± 0.06	0.97 ± 0.03

[a]Microsomes (1.5 mg protein/mL) prepared from rats injected *i.p.* with 2.5 mg cyanamide/100 g body wt/0.1 mL saline or an equivalent volume of saline were incubated with 0.1 mM 4-HN for 1 hr at 37°C.

[b]Units of measurements and statistical analysis are the same as those in Table 1, except that ALDH was analyzed using Student's *t*-test (N = 3 rats).

[c]nd, Not detected.

reduced/mg protein, which was 20% of that using 1.0 mM hexanal as substrate (53.8 nmol NADH reduced/mg protein). The activity was lost in microsomes incubated with iron and ascorbate for 1 hr. 4-HN at 0.1 mM did not significantly increase TBARS in microsomes from cyanamide-treated rats as compared to those from controls (Table 2). The Cyt-P450 and the activities of Cyt-*c*-Rx and GSH *S*-Tr were slightly, but not significantly, decreased in microsomes from cyanamide treated rats. The 4-HN-induced loss of G6Pase activity in microsomes from cyanamide-treated rats was twice that in control rats. When 4-HN was added at 25 μM to the microsomes, none of the above differences was observed (data not shown).

DISCUSSION

The present studies investigated the relationship between enzyme inactivation and membrane lipid peroxidation in rat hepatic microsomes. We have studied both lipid peroxidation and enzyme inactivation induced by iron and ascorbate, as well as by 4-HN. We studied several enzymes located at different membrane sites and examined the effect of antioxidants; others had previously either examined only a single enzyme (11,20) or did not use antioxidants (21). In our studies, particular emphasis was placed on G6Pase and ALDH. G6Pase has long been known as an integral structural membrane protein (22) and also for its susceptibility to lipid peroxidation (5,22). By contrast, the sensitivity to lipid peroxidation of microsomal ALDH was discovered only recently (11,23).

In microsomes incubated with ferric sulfate and ascorbate, there was a marked increase in TBARS and a decrease in G6Pase activity. Cyt-*c*-Rx was not significantly affected by iron and ascorbate. These findings are in agreement with those of Itoh *et al.* (21). The study further showed that iron and ascorbate strongly decreased ALDH activity and Cyt-P450, but not GSH *S*-Tr, in hepatic microsomes. The time-course study showed that changes in these enzymes were concurrent with the progression of lipid peroxidation. ALDH was previously shown to be inactivated rapidly by carbon tetrachloride in hepatocytes (11), and in liver of rats injected with diethylmaleate alone and diethylmaleate followed by bromotrichloromethane (23). The present study demonstrated that ALDH was a more sensitive marker of microsomal lipid peroxidation induced by iron and ascorbate than was G6Pase.

The susceptibility of enzymes to iron and ascorbate inactivation appeared to depend upon the location of the enzyme in the microsomes. For instance, G6Pase is tightly associated with, and deeply embedded into, the hydrophobic core of the microsomal membrane (22), while Cyt-*c*-Rx (21) and GSH *S*-Tr (24) are located closer to the cytosolic surface of the microsomal membrane. Like G6Pase, the microsomal ALDH is tightly bound to the membrane and cannot be solubilized by sonication or extensive washing (9,10). Inactivation of G6Pase and ALDH thus indicates that the microsomal membrane was extensively disrupted during incubation with iron and ascorbate. Interestingly, the nonthiol antixoidants BHT and DPPD inhibited lipid peroxidation and protected enzymes against inactivation by iron and ascorbate. Similarly, dietary vitamin E protects hepatic Cyt-P450 peroxidase against *in vivo* lipid peroxidation induced by methyl ethyl ketone peroxide (25). Although the microsomal enzyme inactivation occurred rapidly and concurrently with lipid peroxidation, our data do not conclusively demonstrate that the former was caused by the latter.

SH enzymes are known to be susceptible to oxidative damage (1). While microsomal GSH *S*-Tr (24) and Cyt-*c*-Rx (17) do not depend upon SH groups for activity, the SH groups essential for activities of G6Pase (21,26), ALDH (27) and Cyt-P450 (28) may be modified readily by reactive substances produced in microsomes by iron and ascorbate. This is suggested by the observation that addition of GSH to microsomes not only decreased TBARS but also protected these enzymes against inactivation by iron and ascorbate. The activity of hepatic ALDH is highly correlated with the level of hepatic GSH in the rat (23).

In the present study we also investigated whether prior inactivation of ALDH by cyanamide accentuated inactivation of enzyme in microsomes incubated with 4-HN. Mitchell and Petersen (12,13) have reported a single ALDH isozyme in rat liver microsomes, and the purified ALDH does not metabolize 4-HN. However, we found that microsomal preparations had low, but clearly detectable,

ALDH activity using 4-HN as substrate (20% the activity using hexanal). This activity was not due to contamination from cytoplasmic isozyme since our microsomes were thoroughly washed and the activity was measured under greatly diluted condition (to 0.10 mg protein/mL of assay mixture). 4-HN added at 0.1 mM strongly inactivated G6Pase without enhancing lipid peroxidation in microsomes devoid of ALDH activity as a result of injection of rats with cyanamide. The inactivation was greatly decreased in microsomes containing active ALDH and its cofactor, NAD^+. However, there was no inactivation of G6Pase when 4-HN was added to microsomes at 25 μM (equivalent to 17 nmol/mg protein), a concentration that is 5- and 85-fold greater than that produced in rat liver microsomes incubated with carbon tetrachloride and ADP-Fe, respectively (6). Thus, our data suggest that inactivation of G6Pase by 4-HN *in vivo* is questionable, although Benedetti *et al.* (29) have attributed the inactivation of the enzyme in microsomes to long-chain aldehydes produced from lipid peroxidation. Since TBARS were not increased in microsomes incubated with 4-HN, the inactivation of enzymes by 4-HN was not attributed to stimulation of lipid peroxidation. Because 4-hydroxyalkenals, such as 4-HN, require no prior activation by the mixed function oxidase system (28), 4-HN may inactivate enzymes through its well-established reactivity with the SH groups of the enzymes. However, the following reasons show that 4-HN does not act as a major second cytotoxic messenger of lipid peroxidation: i) G6Pase was only inactivated by a rather high concentration of 4-HN; ii) the high reactivity of 4-HN with GSH, which is of the order of 10 mM in liver, renders 4-HN unlikely to damage cellular components that are remote from microsomes (10); and iii) the presence of ALDH in mitochondria, microsomes and cytosol (9,10) provide stringent protection of liver against toxic aldehydes. G6Pase may not be the most sensitive target of 4-HN toxicity. Eckl and Esterbauer (6) have recently shown that 4-HN is genotoxic at submicromolar concentrations to rat hepatocytes. It also cannot be excluded that the concentration of 4-HN in the microsomal membrane microenvironment may be locally high enough to cause enzyme inactivation.

In summary, iron and ascorbate induced microsomal enzyme inactivation and membrane lipid peroxidation, both of which were inhibited by antioxidants. Enzymes tightly bound to microsomal membrane, particularly the SH enzymes, were most susceptible to oxidative damage, and ALDH was an outstanding example. Inactivation of ALDH by cyanamide injection of rats showed that 4-HN and other aldehydic products of lipid peroxidation exacerbated inactivation of certain liver enzymes. However, 4-HN appears unlikely to play a significant role in enzyme inactivation because it inactivated G6Pase only at very high concentrations and it did not stimulate lipid peroxidation. By contrast, lipid peroxyl radicals may play a dominant role in microsomal enzyme inactivation induced by iron and ascorbate. This was evidenced by the protection of microsomes by chain-breaking antioxidants.

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Nitric Oxide, an Inhibitor of Lipid Oxidation by Lipoxygenase, Cyclooxygenase and Hemoglobin

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The present study demonstrated that nitric oxide, which is an important mammalian metabolite, can inhibit oxidation by lipoxygenase, cyclooxygenase and hemoglobin. The inhibition is manifested as a lag-phase that is reversible. The inhibitory effect of nitric oxide on lipoxygenase and cyclooxygenase seems to derive from i) the capability of •NO to reduce the ferric enzyme to the ferrous form, which is inactive; ii) competition for the iron site available for exogenous ligands; and iii) the radical scavenging ability of the nitroxide radical. Nitric oxide may act as a modulator of the arachidonic acid cascade and in the generation of oxygen-active species.
Lipids 27, 46-49 (1992).

Mammalian cells can synthesize nitric oxide (•NO) (1). Nitric oxide is synthesized from L-arginine by vascular endothelial cells (2), macrophages (3), the adrenal gland (4), and brain cytosol (5). The main function of •NO seems to be to stimulate the soluble guanylate cyclase to elevate cyclic guanidine monophosphate (cGMP) (6). The system activates a variety of biological functions which include endothelium-dependent relaxation, cytotoxicity of phagocytic cells and cell-to-cell communication in the central nervous system (7,8). Nitric oxide plays an important role in the cardiovascular system, not only by controlling the vascular tone but also by inhibiting platelet aggregation and platelet adhesion (9,10). •NO is thought to activate soluble guanylate cyclase through interaction with the iron of the enzyme's heme (11). In the same way •NO interacts with hemoglobin (12,13), the FeS cluster of nitrogenase (14), and FeS center proteins in *Clostridium botulinum* (15).

Inhibition of platelet aggregation by •NO is also thought to occur through stimulation of guanylate cyclase (7). Platelet aggregation is known to be affected by cyclooxygenase, which activates the arachidonic acid cascade and the production of thromboxane A_2 (TXA_2) (16). Lipoxygenase, which is present in platelets, leukocytes and many other cells, is another enzyme which affects the arachidonic acid cascade and converts arachidonic acid to leukotrienes. Leukotrienes are potent eicosanoids which, *inter alia*, cause smooth muscle contraction, bronchoconstriction, increased vascular permeability, and edema (17).

Reduced iron (Fe^{2+}), complexed by anions and especially by protoporphyrin to form heme, has a high binding affinity for, and reactivity with, •NO. The •NO reacts with the Fe^{2+} ion of heme or other complexes to yield the paramagnetic •NO/Fe complex species (13,18). Most recently, we found that nitric oxide adducts with ferrous complexes affect the Fenton reaction and prevent hydroxylation of benzoate to monohydroxy compounds (19).

Lipoxygenase and cyclooxygenase are enzymes which contain iron at the active site of the molecule (20,21). Nitric oxide, by interacting with iron at the active site, may inactivate these enzymes. The aim of this study was to demonstrate that nitric oxide acts as an inhibitor of lipid oxidation by lipoxygenase, cyclooxygenase and hemeproteins, such as hemoglobin.

MATERIALS AND METHODS

Ascorbic acid, all-*trans*-β-carotene, linoleic acid, Tween-20, diethylenetriaminepentaacetic acid (DETA), hemoglobin (bovine), and soybean lipoxygenase type I were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium nitrite was obtained from BDH Ltd. (Poole, U.K.).

Nitric oxide was prepared according to the method of Bunton *et al.* (22). Ascorbic acid (0.1 M) in acetate buffer, pH 4.5, was reacted with sodium nitrite (0.2 M) under a stream of N_2. Nitric oxide generated by this reaction was transferred by the N_2 stream at a rate of 6 mL/min (corresponding to 480 nmole/min •NO) through a tube which contained the lipoxygenase, microsomal cyclooxygenase or hemoglobin in phosphate buffer, pH 7.3, for 2 min for lipoxygenase and cyclooxygenase, and for 5 min for hemoglobin, followed by a 2-min stream of only N_2. The same procedure of •NO flushing was applied to distilled water and phosphate buffer alone. Nitric oxide was quantified by a difference-spectrometric method which is based on the rapid conversion of oxyhemoglobin to metmyoglobin by •NO (23).

Bovine seminal vesicles were obtained fresh from a slaughter house. Seminal vesicle microsomes were prepared as described previously (24). Briefly, fresh seminal vesicles (250 g) taken from bovines were homogenized with a polytron in 0.25 sucrose/5 mM Tris buffer, pH 7.4, at 4°C. After removing the nuclei erythrocytes, mitochondria and cells by centrifugation, the microsomal fraction was obtained by centrifugation at 105,000 × *g* for 60 min. The sedimented microsomes were resuspended in fresh sucrose/buffer and recentrifuged. The final pellet was used for our experiments. The microsomal pellet could be kept for several weeks at −20°C without any considerable loss of activity. Microsomal cyclooxygenase activity was determined by the β-carotene bleaching assay.

β-Carotene/linoleate oxidizing activity was assayed colorimetrically (25). The technique consists of following the decrease in absorbance at 460 nm in the cuvette of a double-beam recording spectrophotometer. The test sample contained 1.5 mL of buffered carotene linoleate mixture at pH 7.3, 0.1-0.4 mL active fractions, and distilled water to a final volume of 2.0 mL. Concentrations in the initial reaction mixture were as follows: β-carotene, 14 μM;

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Abbreviations: cGMP, cyclic guanidine monophosphate; Cyclo, cyclooxygenase; DETA, diethylenetriaminepentaacetic acid; Hb, hemoglobin; LPO, lipoxygenase; •NO, nitric oxide; •NO-Cyclo, nitric oxide cyclooxygenase; •NO-Hb, nitric oxide hemoglobin; •NO-LPO, nitric oxide lipoxygenase; TXA_2, thromboxane A_2.

linoleate, 2 mM; Tween-20, 0.05%; phosphate buffer, pH 7.3, 0.1 M; DETA, 0.5 mM. The blank sample contained all the reagents except β-carotene.

The assay of linoleate diene conjugation was carried out by the method described by Ben-Aziz *et al.* (26). The reaction mixture contained linoleic acid (400 μM), Tween-20 (0.05%) and DETA (0.5 mM) in 0.1 M phosphate buffer at pH 7.3. For microsomal cyclooxygenase, the assay was done after extraction of the lipid fraction and solubilization in cyclohexane. Both assay methods employed a DB Varian recording spectrophotometer (Varian Associates, Palo Alto, CA). Protein was determined by a modified Lowry method (27).

RESULTS AND DISCUSSION

Cyclooxygenase generates prostanoids *via* the stereospecific introduction of oxygen into arachidonic acid, and lipoxygenase synthesizes the oxygenated fatty acids, which are the precursors of leukotrienes. Both enzymes also could oxidize other free fatty acids containing a *cis,cis*-1,4-pentadiene structure, such as linoleic acid (28), or cooxidize β-carotene in the presence of linoleate.

Nitric oxide inhibits lipoxygenase and microsomal cyclooxygenase cooxidation of β-carotene in the presence of linoleate. The inhibition was demonstrated by an activity lag phase of almost 2 and 3.8 min, respectively (Fig. 1). The inhibitory effect of •NO on lipoxygenase activity was also demonstrated by following the accumulation of conjugated dienes during linoleate oxidation (Fig. 2). The formation of the native enzyme is essentially reversible if the •NO-Fe enzymes are treated by evacuation or N_2 flushing for 30 min (Table 1).

Deoxyhemoglobin reacts with nitric oxide to form the •NO-hemoglobin complex. The same compound is obtained when methemoglobin is reacted with nitric oxide (Fig. 3), consistent with earlier results by us and others (28). Hemoglobin partially simulated the oxidative activities of cyclooxygenase, a known hemeprotein. Hemeproteins in the presence of trace hydroperoxides are known to catalyze polyunsaturated fatty acid oxidation (29). Hemoglobin oxidizes linoleate, during which reaction β-carotene cooxidizes and conjugated dienes are formed. However, •NO-hemoglobin lost this activity (Fig. 4). •NO-hemoglobin, like lipoxygenase and cyclooxygenase, was reactivated by 30-min gassing of the complex (results not shown).

We have already shown that nitric oxide prevents the prooxidative effects of myoglobin or Fe^{2+}-cysteine toward lipid peroxidation in model systems (30,31). •NO-myoglobin and •NO-Fe^{2+}-cysteine, by themselves, act as

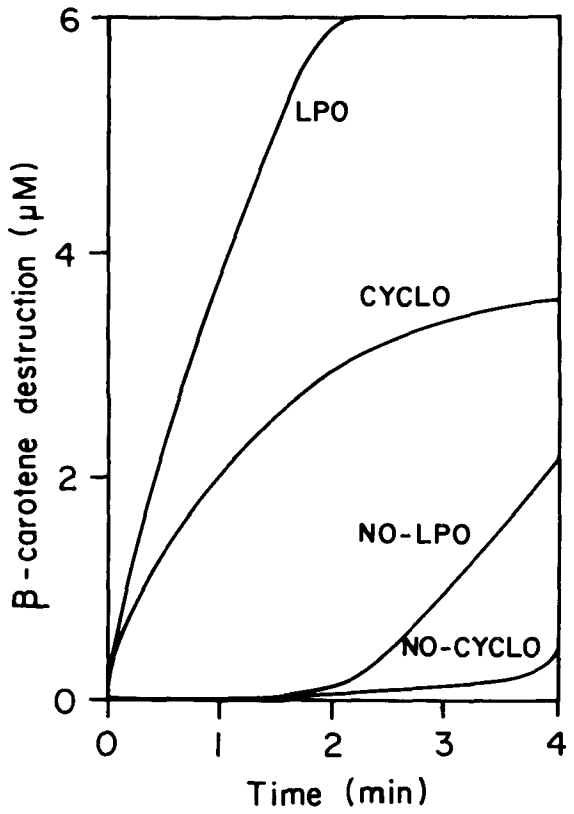

FIG. 1. Coupled oxidation of β-carotene/linoleate by lipoxygenase (LPO) (80 U/mL) and microsomal cyclooxygenase (Cyclo) (320 μg protein/mL) before and after treatment (2 min) with nitric oxide (480 nmole/min, followed by 2 min of N_2 flushing).

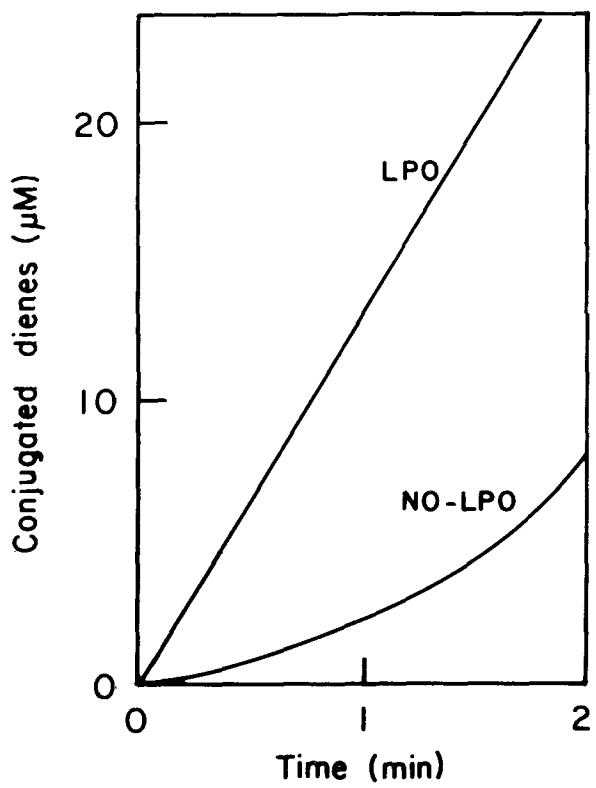

FIG. 2. Linoleate peroxidation determined by accumulation of conjugated dienes, as affected by lipoxygenase (80 U/mL) before and after treatment with nitric oxide (•NO flushing, see Fig. 1).

TABLE 1

The Effect of N_2 Flushing on •NO-LPO and •NO-Cyclo Reactivation[a]

Treatment N_2 flushing (min)	•NO-LPO	•NO-Cyclo
	% reactivation	
2	0	0
15	42	65
30	90	95

[a]LPO and Cyclo activity were determined by the β-carotene/linoleate method (see Materials and Methods).

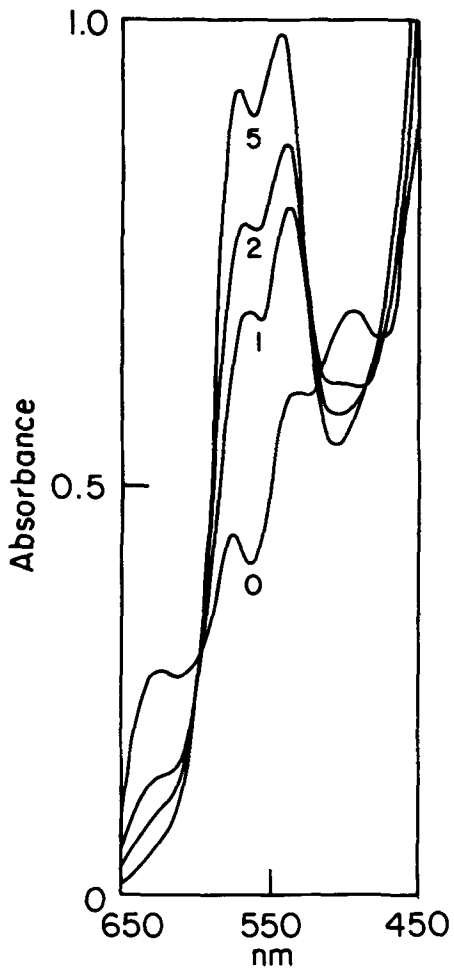

FIG. 3. Spectral changes of methemoglobin (1.5 μg/mL) after interaction with nitric oxide, and generation of the nitric oxide-hemoglobin complex after 0, 1, 2 and 5 min of incubation (•NO flushing, see Fig. 1).

antioxidants. The mechanism we proposed for the antioxidative effect of the nitric oxide compounds was based on the rapid reaction of the nitroxide radical with free radicals involved in lipid peroxidation (32). Recently, Nilsson *et al.* (33) also demonstrated that nitroxide spin labels act as inhibitors of lipid peroxidation.

ESR spectra of •NO-hemoglobin were reported some

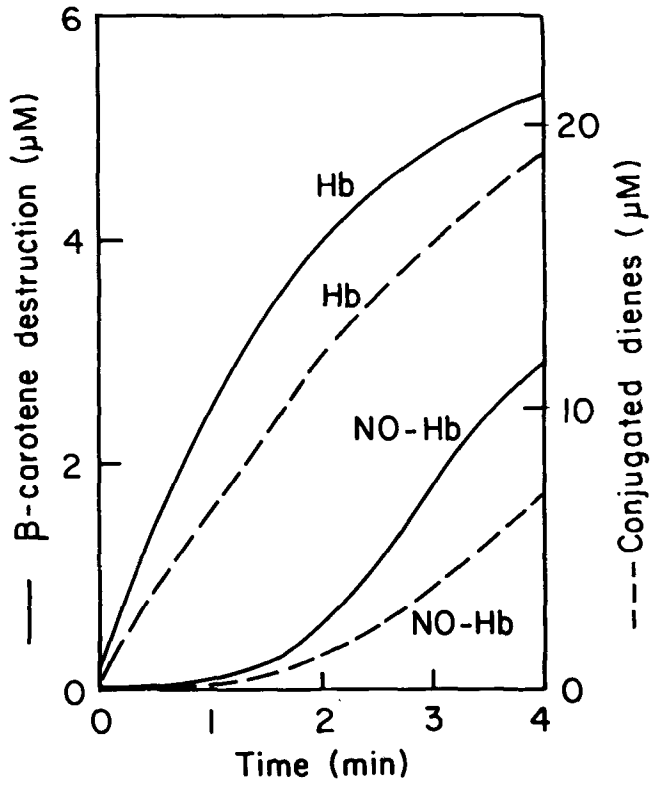

FIG. 4. Coupled oxidation of β-carotene/linoleate and linoleate peroxidation determined by accumulation of conjugated dienes as affected by hemoglobin (Hb) (2.2 μg/mL) before and after (5 min) treatment with nitric oxide (480 nmole/min, followed by 2 min of N_2 flushing).

time ago (12,13). Cyclooxygenase probably also forms a •NO-heme adduct. Lipoxygenase, which is a non-heme protein, contains iron in the active center; the ESR spectrum of •NO-lipoxygenase has been described (34,35).

The binding of linoleic acid to lipoxygenase is competitive with that of •NO at pH 9, but not at pH 7, suggesting that the affinity of ferrous lipoxygenase to •NO at pH 7 is high. In lipoxygenase there seems to be only one coordination site available at the ferrous ion for exogenous ligands (35). It is also important to note that the affinity between •NO and iron-heme is very high (36). An iron-heme without the proximal base reacts with •NO at a rate constant of 3×10^8 M^{-1} S^{-1} (37). These results suggest that the rate constant of •NO with iron-heme and non-heme compounds, especially with more open iron complexes, reaches a diffusion controlled limit.

The inhibitory effect of nitric oxide toward lipoxygenase and cyclooxygenase may stem not only from the radical scavenger ability of the nitroxide radical, but also from the capability of •NO to reduce the ferric-active enzyme to the ferrous-inactive form. The reducing capability of •NO was demonstrated with methemoglobin forming the •NO-ferrous hemoglobin. The ferrous forms of lipoxygenase and cyclooxygenase are inactive (28,38) and need to be activated to the ferric form by traces of hydroperoxides or hydrogen peroxide. The availability of a free coordination site is a stringent requirement for coordinative binding of H_2O_2 or hydroperoxides to ferrous or ferric heme proteins (39). •NO liganded to the coordination site

of iron in lipoxygenase or to the six coordination sites of iron cyclooxygenase may prevent the hydroperoxides or H_2O_2 from being bonded in a coordinating manner to the ferrous enzymes. However, it does not prevent the interaction of iron valence electrons from tg and t2g orbitals with H_2O_2 or hydroperoxide valence orbitals (28,39). These changes by •NO may decrease the activation rate of the enzymes from the ferrous to the ferric form by hydroperoxides, and explain the lag phase in •NO-enzyme activities, as shown in our study. We must consider that basal synthesis of •NO *in vivo* is continuous upon stimulation and therefore may afford extended inhibition as long as the stimulatory effect persists.

We conclude that, in addition to its stimulatory effect on soluble guanylate cyclase, nitric oxide seems to affect oxidative reactions by: i) modulating enzymes containing transition metals; ii) chelating "free" iron ions in a form which prevents or reduces the catalytic effects of the metal; and iii) scavenging free radicals. Generally, these reactions of nitric oxide may serve to protect cells from the cytotoxicity of active oxygen species, but •NO may also act as a cytotoxic compound (40,41). Since •NO has a very short life span, its overall effect seems to be dependent upon its site of biosynthesis, its concentration and the simultaneous presence of compounds containing transition metals.

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Formation of Formaldehyde and Malonaldehyde by Photooxidation of Squalene

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Formaldehyde and malonaldehyde were identified upon exposure of squalene to ultraviolet (UV) irradiation at 300 nm. Formaldehyde was derivatized by reaction with cysteamine to form thiazolidine; malonaldehyde was derivatized by reaction with *N*-methylhydrazine to produce *N*-methylpyrazole. The derivatives were subsequently analyzed with a gas chromatograph equipped with a fused silica capillary column and a nitrogen/phosphorus detector. The levels of formaldehyde and malonaldehyde produced increased with irradiation time. The amount of formaldehyde produced reached a maximum of 3.40 nmol/mg squalene after 7 hr irradiation; the maximum amount of malonaldehyde generated, 0.92 nmol/mg, was found after 5 hr of irradiation. Prior to this study, formaldehyde had not been reported as a photoproduct of squalene. Acetaldehyde and acetone were also detected in the irradiated squalene, which may be formed *via* a 6-methyl-5-hepten-2-one intermediate. 6-Methyl-5-hepten-2-one can also undergo breakdown to form malonaldehyde.
***Lipids* 27, 50–53 (1992).**

Lipid peroxidation, which has been linked with carcinogenicity, mutagenicity, aging, and cytotoxicity, involves production of aldehydes from lipids (1–3). Among the aldehydes formed, malonaldehyde (MA) has received the most attention. Studies have shown, for example, that mice developed carcinomas of internal organs when MA was applied to their shaved backs (4). Other studies have found MA to be mutagenic (5).

Numerous studies on the formation of aldehydes, including formaldehyde, acetaldehyde, acrolein, MA, and 4-hydroxynonenal, from fatty acids have been reported (6,7). The recent development of a highly sensitive gas chromatographic method has made the measurement of these reactive aldehydes possible (8,9).

Excessive exposure of human skin to ultraviolet (UV) radiation causes peroxidation of skin lipids (10,11). Therefore, squalene, a major lipid component of skin, may generate carbonyl compounds by reaction with UV light. Previous studies have demonstrated that a correlation exists between the amount of lipid peroxides formed and the total amount of squalene exposed to UV irradiation (12,13).

In the present study, squalene was exposed to 300 nm UV light, to stimulate exposure to sunlight, and the levels of HCHO and MA were determined by gas chromatography. In addition, the mechanisms by which HCHO and MA are formed in lipid systems were examined.

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; MA, malonaldehyde; 6-MHO, 6-methyl-5-hepten-2-one; NPD, nitrogen/phosphorus detector; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Materials. Squalene and 6-methyl-5-hepten-2-one (6-MHO) were purchased from Sigma Chemical Co. (St. Louis, MO). Cysteamine HCl, thiazolidine, sodium dodecyl sulfate (SDS), 2-methylpyrazine, and tributylamine were obtained from Aldrich Chemical Co. (Milwaukee, WI). *N*-Methylhydrazine was purchased from Fluka Chemical Co. (Ronkonkoma, NY). Dichloromethane and chloroform (reagent grade) were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ).

Standards of 2-alkylthiazolidines were synthesized according to a method published earlier (14). The purity of the standards determined by gas chromatography varied between 99.1 and 99.9%. *N*-Methylpyrazole was synthesized by a method previously reported (9) and was 99.5% pure. Stock solutions of the internal standards were prepared by adding 100 mg of tributylamine to 100 mL of chloroform, and 15 mg of 2-methylpyrazine to 100 mL dichloromethane.

Photoirradiation of squalene. Pyrex tubes each containing 172 mg of squalene, 10 mL of deionized water, and 4 mL of 5% SDS were irradiated for various time periods in a Rayonet RPR-100 Chamber Reactor. Eight lamps (300 nm wavelength) were fitted in the reactor. Several pyrex tubes containing the above mixtures were wrapped with aluminum foil and stored in a freezer to serve as controls. The irradiated and the not irradiated sample were then divided each into two equal portions for derivatization.

Photoirradiation of 6-MHO. Pyrex tubes each containing 85.5 mg of 6-MHO, 10 mL of deionized water, and 4 mL of 5% SDS were treated following the same procedure as for squalene, except that the samples were irradiated for 5 hr only.

Derivatization of formaldehyde. Cysteamine (0.6 mmol) was added to a portion of the above solutions, and the pH was adjusted to 8.5 with 1N NaOH. The mixture was stirred at 25°C for 1 hr and then extracted with approximately 12 mL of chloroform using a liquid-liquid extractor for 5 hr. The extract was then brought to a final volume of 10 mL, and 400 μL of tributylamine stock solution was added as internal standard for gas chromatography (GC).

Derivatization of malonaldehyde. N-Methylhydrazine (40 μL) was placed into the second portion of the above solutions, and the mixture was stirred for 1 hr at 25°C. The derivatives were then extracted with approximately 12 mL of dichloromethane for 3 hr using a liquid-liquid continuous extractor. Saturated NaCl was added to the aqueous layer before extraction to prevent emulsion formation. The extract was then adjusted to a final volume of 10 mL, and 150 μL of the stock solution of 2-methylpyrazine was added as an internal standard.

Instrumental analysis. A Hewlett-Packard (HP) Model 5890A gas chromatograph (Palo Alto, CA) equipped with a nitrogen/phosphorus detector (NPD) and a 30 m × 0.25 mm i.d. × 0.25 μm film thickness bonded phase DB-1 fused silica capillary column (J & W Scientific, Folsom, CA)

was used for the quantitation of the thiazolidine derivative of HCHO. The GC peak areas were integrated by a Spectra-Physics Chromjet Integrator (San Jose, CA). Oven temperature was held at 80°C for 2 min and then programmed to 200°C at 8°C/min. Injector and detector temperatures were 250°C and 300°C, respectively. Linear velocity of helium carrier gas was 25.5 cm/sec at a split ratio of 55:1.

An HP 5880 GC equipped with an NPD and a 30 m × 0.25 mm i.d. × 0.25 μm film thickness bonded phase DBWAX fused silica capillary column (J & W Scientific) was used for the analysis of the *N*-methylpyrazole derivative of MA. The GC peak areas were integrated by an HP 3390A integrator. The oven temperature was held at 35°C for 2 min and then programmed to 190°C at 4°C/min. Injector and detector temperatures were 250°C and 300°C, respectively. Linear velocity of helium carrier gas was 35.5 cm/sec at a split ratio of 30:1.

An HP model 5890 GC interfaced to a VG Trio II mass spectrometer with a VG 11-250 computer data system was used for mass spectrometric identification of the GC components. The ionization voltage was 70 eV, and the ion source temperature was 150°C. The column and oven conditions for gas chromatography/mass spectrometry were as described for the GC/NPD analysis.

RESULTS AND DISCUSSION

Figure 1 shows a typical gas chromatogram of the thiazolidine derivatives obtained from squalene after ultraviolet (UV) exposure. In addition to HCHO, other monocarbonyl compounds, such as acetaldehyde and acetone, were detected as 2-methylthiazolidine (peak 4) and 2,2-dimethylthiazolidine (peak 5), respectively. The two peaks after peak 8 are SDS breakdown products.

The *N*-methylhydrazine derivative of MA was detected as *N*-methylpyrazole. *N*-Methylhydrazine reacts with α,β-unsaturated aldehydes such as acrolein to form *N*-methylpyrazoline. Acrolein, however, was not detected in this study.

The levels of HCHO and MA formed after various irradiation times are presented in Figure 2. The levels of these aldehydes were increased by UV irradiation. The maximum amount of HCHO was formed after 7 hr of irradiation and was four times the control level. The maximum amount of MA formed was ten times the control level and occurred after 5 hr of irradiation. The results obtained for MA were consistent with those reported previously (15).

In the present study, formaldehyde was detected as a peroxidative product of squalene. Formaldehyde has previously been reported to crosslink with DNA and nucleoproteins (16–18), possibly leading to carcinogenicity. In addition, formaldehyde has been implicated as an animal and human carcinogen (19–22) and has been shown to cause dermatitis in rats (23).

Other volatile aldehydes observed are shown in Table 1. After 7 hr of irradiation, acetaldehyde was the major aldehyde formed, followed by HCHO, acetone, and MA. In the control sample, HCHO and acetone were formed in relatively low amounts (less than 1.3 nmol/mg squalene) while acetaldehyde was produced in relatively high amounts (1.70 nmol/mg squalene). The formation of acetone as a product of lipid peroxidation *in vivo* has been reported (24). Studies have also associated the accumulation of acetone with diabetes mellitus (25). The precursor of acetone in these studies, however, is still unclear.

The presence of 6-MHO was observed only in the irradiated squalene samples, suggesting that 6-MHO is a possible intermediate in the formation of the low-molecular-weight volatile carbonyl compounds from squalene. The amount detected was approximately 120

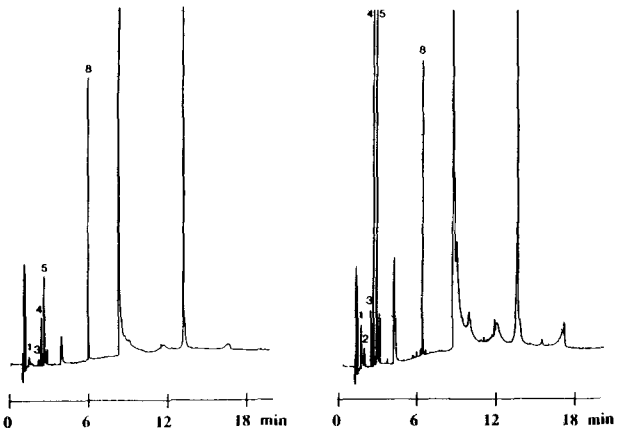

FIG. 1. Gas chromatogram of the cysteamine derivatives of volatile aldehydes from non-irradiated squalene (left) and irradiated squalene at 300 nm (right). Peak 3, thiazolidine; 4, 2-methylthiazolidine; 5, 2,2-dimethylthiazolidine; 8, internal standard.

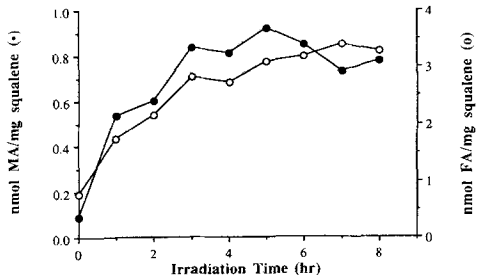

FIG. 2. Production of malonaldehyde (MA) (●) and formaldehyde (HCHO) (○) during UV irradiation of squalene.

TABLE 1

Volatile Peroxidation Products Recovered from Squalene After 7 hr of Irradiation

Compound	Squalene (nmol/mg)[a]	
	Dark	Irradiated
Formaldehyde	<1.30	3.40 ± 0.24
Acetaldehyde	1.70 ± 0.57	10.37 ± 2.42
Acetone	<1.30	2.51 ± 0.01
Malonaldehyde	<0.12	0.73 ± 0.02

[a]Values are means of three replicates ± standard deviation.

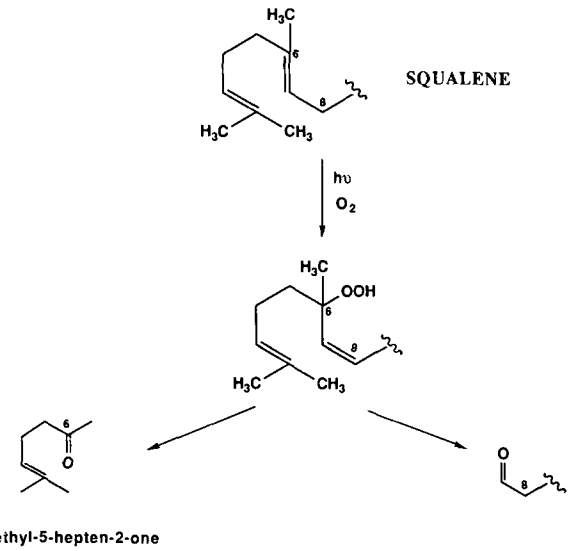

FIG. 3. Proposed formation of 6-methyl-5-hepten-2-one from squalene upon UV irradiation.

TABLE 2

Volatile Peroxidation Products Recovered from 6-Methyl-5-hepten-2-one (6-MHO) After 5 hr of UV-Irradiation

	6-MHO (nmol/mg)[a]	
Compound	Dark	Irradiated
Formaldehyde	<1.30	4.14
Acetaldehyde	1.84	4.22
Acetone	<1.30	3.95
Malonaldehyde	0.65	1.28

[a]Values are means of two replicates.

μg after 6 hr of UV irradiation. The proposed mechanism for the formation of 6-MHO from squalene is shown in Figure 3. The formation of 6-MHO results from the abstraction of a proton from the C-8 carbon of squalene. This is followed by the generation of a 6-hydroperoxide intermediate, which undergoes cleavage between the C-6 and C-7 carbon-carbon bond.

Samples with 6-MHO were irradiated by UV for 5 hr to examine the formation of volatile carbonyl compounds from 6-MHO. The results are shown in Table 2. Among the carbonyl compounds quantitated, acetaldehyde had the highest level in the control samples. The amounts of HCHO, acetaldehyde, and acetone produced in irradiated samples were significantly higher than those of MA. Similar to samples from irradiated squalene, acrolein was also not detected in the irradiated 6-MHO samples. The observation further indicates that 6-MHO is a possible intermediate of volatile carbonyls.

In our study, MA was formed from a compound with only one double bond. Earlier studies proposed the formation of MA *via* a cyclic peroxide (26). Later, MA was proposed to form through a bicyclic endoperoxide intermediate (27). This widely accepted mechanism requires at least three methylene-interrupted double bonds for the formation of MA. However, our results with 6-MHO imply that compounds with one double bond could also produce MA. There may be an alternative route involving compounds with less than three double bonds, as is the case with 6-MHO. Studies on the formation of MA from precursors with one double bond have been reported (6,28). Other studies have demonstrated the formation of malonaldehyde from unsaturated carbonyl compounds (29).

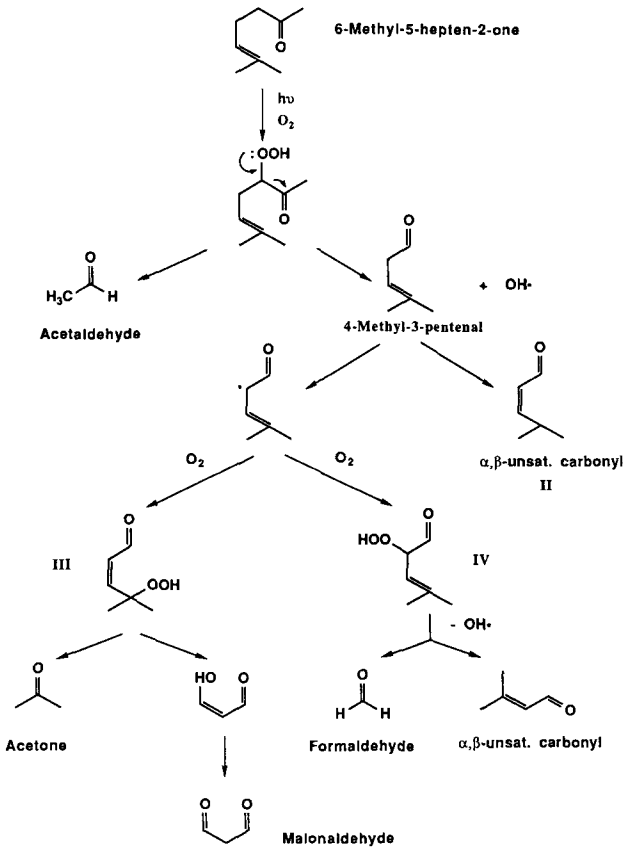

FIG. 4. Proposed formation of volatile carbonyls from 6-methyl-5-hepten-2-one upon UV irradiation.

The proposed mechanism of formation of the various carbonyl compounds identified in this study is shown in Figure 4. The formation of acetaldehyde involves the initial abstraction of a proton from 6-MHO. The resulting hydroperoxide may then undergo decomposition to form acetaldehyde and 4-methyl-3-pentenal intermediate **I**. 4-Methyl-3-pentenal was not detected as it may have undergone further reaction to form HCHO, MA, and acetone, or it may have isomerized to **II**, an α,β-unsaturated aldehyde. Similarly, abstraction of a proton may occur on 4-methyl-3-pentenal to form either 4-hydroperoxide **III** or 2-hydroperoxide **IV** intermediates. Subsequently, intermediate **III** undergoes chain-scission type reactions to form acetone and malonaldehyde (30); and intermediate **IV** would form HCHO.

Although squalene is one of the major components of the skin, composing 5% of the skin surface lipids (31), few reports have appeared on the mechanisms by which reactive aldehyde may form upon lipid peroxidation. Some

studies have demonstrated that squalene is the major source of lipid peroxide in the skin surface (10,11). Further studies will be necessary to establish the relationship between lipid peroxidation and the formation of toxic aldehydes that can cause skin damage.

ACKNOWLEDGMENT

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Acute Toxicity of *trans*-5-Hydroxy-2-nonenal in Fisher 344 Rats

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The potential toxicity of *trans*-4-hydroxy-2-nonenal (HNE), a product formed *in vivo* during lipid peroxidation, which is also present in foods, was investigated in Fisher 344 rats. Five groups of five male rats each were given by gavage 1000, 300, 100, 30 or 10 mg/kg body weight HNE dissolved in 0.5 mL corn oil. The sixth group, the control, received corn oil alone. Two rats died 6 and 8 hr after being treated with 1000 mg/kg HNE. These two rats showed extensive acute tubular necrosis of the kidney, but had very little liver damage. Diffuse liver cell necrosis was observed in a dose dependent manner in all the rats killed 14 days after treatment, whereas renal change was mild. Interestingly, body weight of the lowest dosage group was significantly higher than that of the control group at termination of the experiment. The results of this study show that HNE has almost the same toxicity as other enals, such as *trans*-2-heptenal, and that kidney and liver are the main organs affected by toxicity of HNE. Although animals may have efficient defense systems, such as glutathione, to detoxify low to moderate dosages of HNE, at high doses of HNE this defense system is overwhelmed, resulting in serious renal and hepatic damage.
Lipids 27, 54-58 (1992).

Some lipid peroxidation products generated by oxygen radicals cause various types of cell injury (1,2). Benedetti *et al.* (2) have suggested that *trans*-4-hydroxy-2-nonenal (HNE) is a major diffusible toxic product of lipid peroxidation; such enals are believed to act as secondary toxic messengers for free radicals (2-6). *In vitro* studies have shown that HNE can damage functional proteins and DNA, cause cell lysis and disturb cellular reproduction (6-10). In addition to its generation during lipid peroxidation, small amounts of HNE are found in various foods and oils (11,12). Some analogous enals of HNE, such as *trans*-2-heptenal, *trans,trans*-2,4-hexadienal and *trans*-4-hydroxy-2-hexenal, which are also present in foods, have been shown to be toxic (13,14). The median lethal dose (LD_{50}) of HNE given intraperitoneally to mice has been estimated to be 68 mg/kg of body weight (15), but the potential toxic effects of oral administration of this enal are not clear. In the present study, the lethal dose of HNE was determined in male Fisher 344 rats. The results of detailed histopathological examination of the major organs are discussed.

MATERIALS AND METHODS

HNE was synthesized according to the method described by Esterbauer *et al.* (16). The purity was greater than 99% as indicated by thin-layer chromatography, high-performance liquid chromatography, and nuclear magnetic resonance spectroscopy. Thirty, seven-week-old male F344 rats were purchased from Charles River Breeding Laboratories (Kingston, NY). Rats were separated into 6 groups and housed 2-3 per cage in polycarbonate cages with hardwood bedding under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12 hr/12-hr light/dark cycle). Before the experiments, animals were fed NIH-07 diet and given tap water for six days. After overnight starvation, each group (consisting of five rats) was given a different treatment. The control group was given 0.5 mL corn oil only. The other five groups were given by gavage 1,000, 300, 100, 30 or 10 mg/kg body weight of HNE dissolved in 0.5 mL corn oil. After the treatment, rats were kept on NIH-07 diet and tap water *ad libitum,* and body weights were measured every three or four days. Animals were sacrificed and a complete autopsy was performed 14 days after gavage. All the main organs were weighed. Most of the organs were collected and fixed in 10% neutral-buffered formalin for routine histological processing and light microscopic evaluation of sections stained with haematoxylin and eosin. The extent of necrosis in the liver and kidney was interpreted according to the criteria described by Mitchell *et al.* (17) and Mizutani *et al.* (18), respectively. The statistical significance was evaluated by Student's *t*-test.

RESULTS

Two rats given 1,000 mg/kg body weight of HNE died six and eight hours after the treatment, respectively. The other animals remained alive until the termination of the experiment. Thus, the LD_{40} of HNE given orally in Fisher rats was estimated as 1,000 mg/kg, although the number of animal used in this study is low. The body

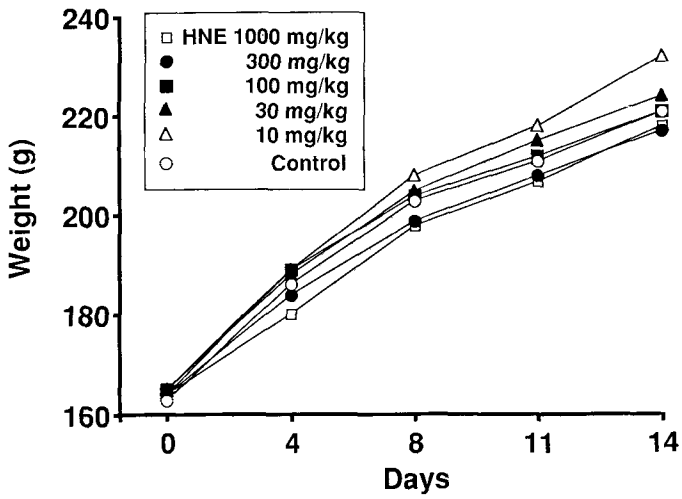

FIG. 1. Body weight curves.

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Abbreviations: HNE, *trans*-4-hydroxy-2-nonenal; LD_{50}, median lethal dose; LD_{40}, 40% lethal dose; NAD, nicotinamide adenine dinucleotide; PUFA, polyunsaturated fatty acid.

TABLE 1

Body Weights at Beginning and at Termination of Experiments

HNE treatment	Beginning	Termination
1,000 mg/kg	164.2 ± 3.8[a] (5)[b]	218.3 ± 12.7 (3)
300 mg/kg	164.4 ± 3.1 (5)	216.6 ± 13.2 (5)
100 mg/kg	165.0 ± 4.6 (5)	221.2 ± 18.0 (5)
30 mg/kg	165.0 ± 6.1 (5)	224.4 ± 8.4 (5)
10 mg/kg	163.8 ± 5.8 (5)	232.0 ± 9.5[c] (5)
Control	163.2 ± 5.1 (5)	220.6 ± 7.3 (5)

[a]Mean ± S.D.

[b]Number in parentheses, number of rats.

[c]Significantly different from control group ($p<0.05$).

TABLE 2

Summary of Histopathology of the Liver and Kidney

	Hepatic necrosis[a]					Renal necrosis[b]				
HNE treatment	0	1+	2+	3+	4+	0	1+	2+	3+	4+
1,000 mg/kg	0	2[c]	2	1	0	0	3	0	0	2[c]
300 mg/kg	0	0	5	0	0	0	5	0	0	0
100 mg/kg	0	2	3	0	0	0	5	0	0	0
30 mg/kg	3	2	0	0	0	1	4	0	0	0
10 mg/kg	5	0	0	0	0	5	0	0	0	0
Control	5	0	0	0	0	5	0	0	0	0

[a,b]The extent of hepatic necrosis and renal tubular necrosis was scored according to the criteria described by Mitchell *et al.* (17) and Mizutani *et al.* (18), respectively. [c]Rats found dead.

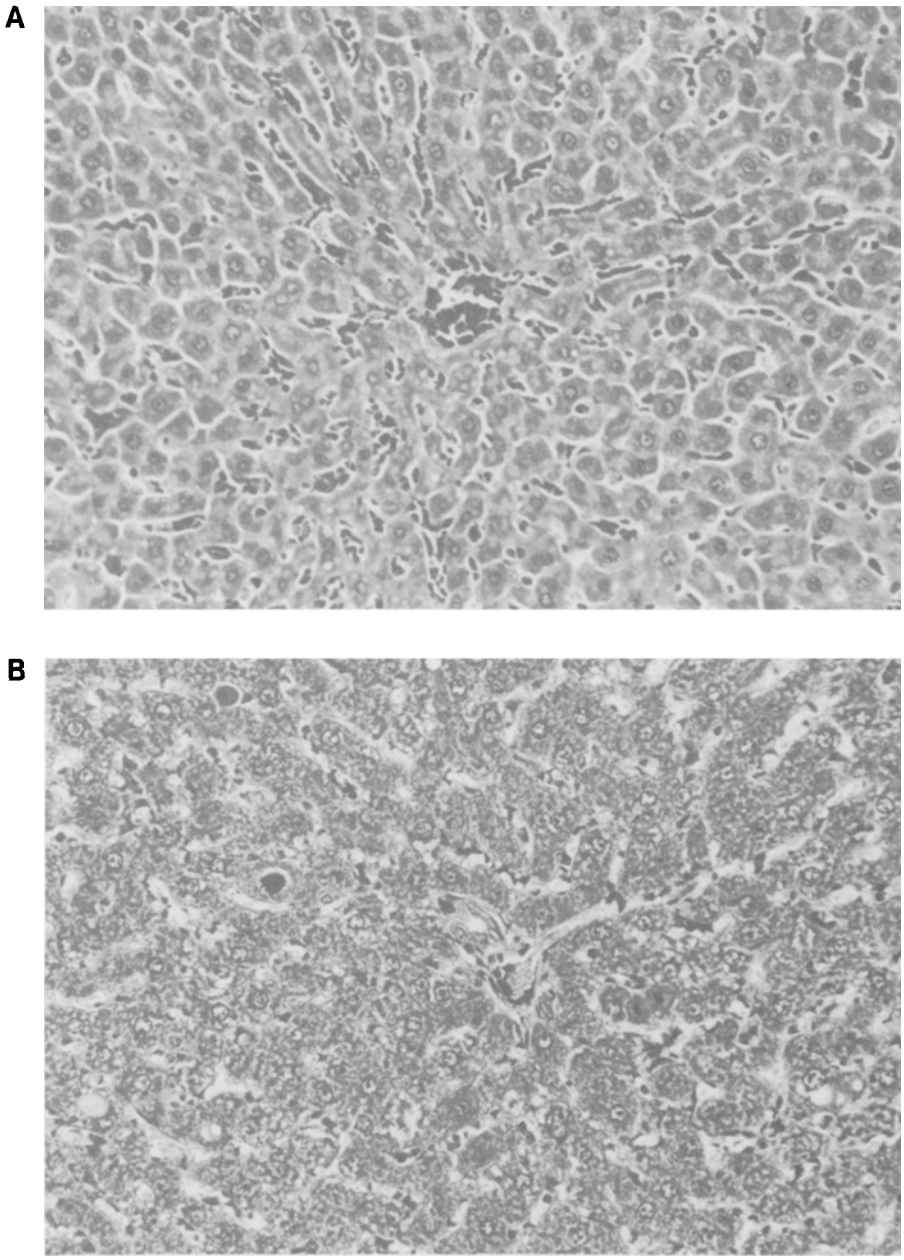

FIG. 2. Histopathology of the liver of a rat treated with 1,000 mg/kg of HNE (a) found dead 6 hr after gavage; and (b) killed at termination. (a) Although marked congestion is seen, mild liver cell necrosis is noticed around the central vein. H-E X250. (b) Moderate liver cell necrosis is seen diffusely and several mitoses are scattered. H-E X250.

weight of the two rats that died early in the experiment markedly decreased after treatment (from 164 g to 140 g and from 168 g to 1442 g, respectively). Body weight curves of the other animals are shown in Figure 1. Body weight gain of rats receiving 1,000 mg/kg and 300 mg/kg of HNE was reduced at the beginning of the experiment, but almost normalized at termination. Rats given 100 mg/kg showed no substantial change in body weight gain compared with the control group. Interestingly, the body weight of rats given 10 mg/kg of HNE was significantly higher than that of the control group ($p<0.05$) at the termination of the experiment, as is shown in Table 1. As for the absolute and relative organ weights of rats killed at the end of the experiment, there were no significant differences between the groups except that the testis of rats given 1,000 mg/kg of HNE was significantly heavier than that of control rats.

Histopathological findings of the liver and kidney are summarized in Table 2. Extensive acute tubular necrosis (moderate to severe) was seen in the kidneys of the dead rats. However, hepatic necrosis was observed only around the central vein of the liver (Fig. 2). The two rats that died early in the experiment showed hemorrhages in the endocardium and thymus, and congestion in the lung, spleen, testis and brain. The other rats given 1,000 mg/kg of HNE revealed moderate hepatic necrosis which was

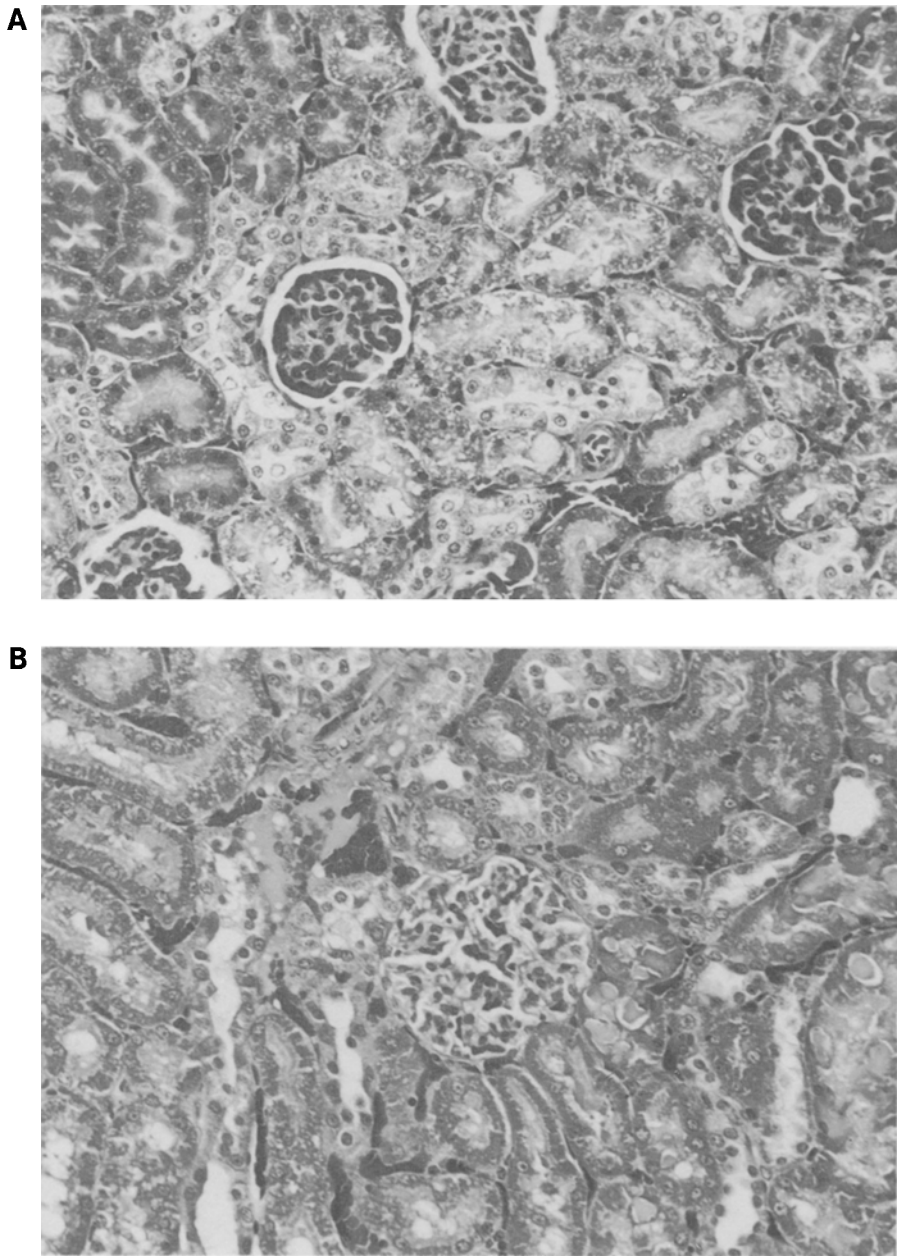

FIG. 3. Histopathology of the kidney of a rat treated with 1,000 mg/kg of HNE (a) found dead 6 hr after gavage; and (b) killed at termination. (a) Moderate to severe acute tubular necrosis is noticed mainly in the paraqcortical area. (b) Mild tubular necrosis is seen in the paracortical area. H-E X250.

seen diffusely, but most extensively, around the central veins. Such changes in the liver were recognized in all treated animals and the extent of lesions depended upon the dosage of HNE. Acute tubular necrosis was not significant in the kidney of rats in all groups killed at the termination of the experiment (Fig. 3). Thus, hepatotoxicity increased dose-dependently, but renal toxicity did not.

DISCUSSION

HNE is an endogenous aldehyde generated during lipid peroxidation, which is also present in small quantity in certain foods consumed by humans (19). Our results suggest that the LD_{40} of HNE given orally to male F344 rats is approximately 1,000 mg/kg body weight, which agrees with the LD_{50} (1,300 mg/kg) of *trans*-2-heptenal given orally to rats (9). However, a larger population of animals and oral doses higher than 1,000 mg/kg would be required for a more accurate estimation. Some autoxidation products of linoleic acid cause hepatotoxicity (20). Segall *et al.* (14) also have reported extensive hepatotoxicity of *trans*-4-hydroxy-2-hexenal, a reactive metabolite from the macrocyclic pyrrolizidine alkaloid senecionine, when it was injected into the portal veins of rats. HNE and *trans*-4-hydroxy-2-hexenal are homologous aldehydic products of lipid peroxidation. The former is produced from the ω-6 polyunsaturated fatty acids (PUFA), linoleic acid, arachidonic acid and γ-linolenic acid, whereas the latter is formed from the ω-3 PUFA, α-linolenic acid and docosahexaenoic acid (21–23). Our study shows for the first time that oral intake of a high dose of HNE can cause lethal renal damage before severe hepatic damage occurs. These results suggest that the secondary autoxidation product administered at a high dose overwhelmed the liver's capacity to serve as the first clearance (24). The time lapse before death occurs depends on the maximal incorporation time into rat kidney of the orally administered radioactive products (20). Tubular necrosis was not extensive in all the kidneys of rats killed at the end of the experiment, perhaps due to recovery from the lesion. Histochemical analysis of renal tubular damage at intermediate time points during the experiment could be useful to determine whether recovery from acute damage occurs. Although the incidence of mortality shows that a threshold for HNE toxicity in the kidney exists, the dose-dependent damage observed in the liver of rats sacrificed at the end of the experiment suggests that the liver is the primary target organ for HNE toxicity. Recently, Oarada *et al.* (25) have found that dietary lipid hydroperoxide causes severe lymphocyte depletion in the thymus. In our study, bleedings were noted in the thymus of the rats that died early in the experiment.

Animals may have adequate defense systems to detoxify aldehydic products of lipid peroxidation, such as HNE (26). HNE is a good substrate of cytosolic nicotinamide adenine dinucleotide (NAD)-dependent alcohol dehydrogenase, aldehyde dehydrogenase and glutathione transferase (27–29). However, the results of this study show that excessive HNE can cause acute and extensive renal or hepatic toxicity. Conjugation involving glutathione has been regarded as a major pathway for detoxification of HNE (5). White and Rees (30) have suggested that HNE toxicity is a result of increased lipid peroxidation which depletes glutathione levels. Measurement of glutathione would be helpful to determine whether depletion of this important antioxidant is necessary for acute renal toxicity of HNE to occur. Our study also shows that low doses of HNE appeared to increase body weight gain, which may be related to regulatory functions in cell division (31). HNE is a homologue of crotonaldehyde which has been known to induce liver tumors in F344 rats (32); therefore, chronic toxicity and carcinogenicity of HNE should be studied to assess its potential role in hepatocarcinogenesis. The results of this study also suggest the possibility of renal tumorigenesis by HNE in rats.

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Preparation and Properties of *gem*-Dichlorocyclopropane Derivatives of Long-Chain Fatty Esters

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Methyl oleate (18:1) and linoleate (18:2) were readily transformed to the corresponding *gem*-dichlorocyclopropane derivatives in high yield, using triethylbenzylammonium chloride as the phase-transfer catalyst in the presence of aqueous NaOH and $CHCl_3$. Reaction of dichlorocarbene with methyl 12-hydroxystearate furnished methyl 12-chlorostearate (49%) and 12-*O*-formylstearate (19%). The hydroxy group in methyl ricinoleate was protected (*O*-tetrahydropyran-2′-yl) prior to dichlorocyclopropanation of the ethylenic bond. Removal of the protecting group allowed the hydroxy group to be converted to a chloride, *O*-acetyl, azido or *O*-formyl function. Treatment of methyl ricinoleate with thionyl chloride, followed by the reaction with dichlorocarbene gave the corresponding 12-chloro-dichlorocyclopropane derivative. The dichlorocyclopropane derivative of oleic acid was transformed to a C_{19} allenic fatty acid when treated with *t*-butyl lithium. However, the remaining dichlorocyclopropane derivatives, containing an additional functional group in the alkyl chain, failed to yield the corresponding allenic derivatives. All derivatives were characterized by a combination of spectroscopic and chromatographic techniques, including infrared, ^1H nuclear magnetic resonance (NMR), and ^{13}C NMR spectroscopy.
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Cyclopropane fatty acids are more commonly found in microorganisms than in plants (1). Lactobacillic acid (*cis*-11,12-methyleneoctadecanoic acid) is commonly found in bacterial lipids (2,3) and mycolic acids are complex fatty acids of high molecular weights occurring in a wide range of micro-organisms containing one or more cyclopropane rings (4). Many eubacteria, such as *Escherichia coli*, modify their membrane phospholipids during the onset of the stationary phase by transforming the ethylenic centers in the fatty acid components to cyclopropane groups (5). *S*-Adenosylmethionine has been shown to effect the formation of the cyclopropane system from unsaturated fatty acid precursors. The function of these unusual fatty acids remains a matter of speculation. It has been postulated that the cyclopropane system preserves the configuration of the ethylenic bond in the fatty acid molecule and prevents autoxidation of the phospholipids in the organism (6,7). More recently, studies on the cloning and manipulation of the *E. coli* cyclopropane fatty acid synthetase gene have been reported (8,9). The relationship between cyclopropane synthetase and the formation of cyclopropane fatty acids by *Proteus vulgaris*, grown under various respiratory conditions, have also been studied (10).

Some long-chain cyclopropane fatty acids, such as dihydrosterculic acid (*cis*-9,10-methyleneoctadecanoic acid), have been identified in the lipid extract of *Euphoria longana* (11) and from *Litchi sinensis* (*Fam. Sapindaceae*) seeds (12). Dihydromalvalic acid (*cis*-8,9-methyleneheptadecanoic acid) has been found in the seed oil of *Hibiscus syracus* (*Fam. Malvaceae*) (13) and from *Brachychiton acerifolium* (*Fam. Sterculiaceae*) (14). A C_{10} cyclopropane fatty acid, *cis*-3,4-methylenedecanoic acid, has been identified in Cascarilla essential oil (15).

Gunstone and Perera (16) have synthesized the entire series of positional isomers of C_{18} cyclopropane fatty esters using the classical Simmons-Smith method (17). A recent modification to the cyclopropanation procedure involves the use of ultrasound to accelerate the rate of reaction (18–21). We report in this paper the preparation of *gem*-dichlorocyclopropane fatty ester derivatives of methyl oleate, linoleate, ricinoleate (12-hydroxy-*cis*-9-octadecenoate) and methyl 12-oxo-*cis*-9-octadecenoate. We have examined the nuclear magnetic resonance (NMR) spectroscopic properties of such dichlorinated cyclopropane fatty ester derivatives and have evaluated the procedure for obtaining allenic fatty esters *via gem*-dichlorocyclopropane intermediates (22). Only a few long-chain allenic fatty acids have been isolated from seed oils so far, and include laballenic acid [(*R*)-5,6-octadecadienoic acid] in the oil of *Leonotis nepetaefolia* (23) and lamenallenic acid (5,6 *trans*-16-octadecatrienoic acid) in the oil of *Lamium purpureum* (24).

A number of procedures have been reported for the synthesis of dichlorocarbene species for reaction with ethylenic bonds to obtain *gem*-dichlorocyclopropane derivatives, which involve anhydrous, homogenous reaction conditions (25,26). Kenney *et al.* (27) have prepared the dichlorocyclopropane derivatives of methyl oleate, elaidate and linoleate using an alkoxide in chloroform or in ethyl trichloroacetate. More recent developments have made use of phase-transfer catalysts, which not only enhance the yield of the products, but also permit reactions to take place under heterogenous conditions (28–31). We have adopted the phase-transfer catalytic method for the preparation of the *gem*-dichlorocyclopropane fatty ester derivatives as presented in Scheme 1, which shows the synthesis of *gem*-dichlorocyclopropane C_{18} derivatives from methyl oleate, linoleate and ricinoleate.

RESULTS AND DISCUSSION

Dichlorocarbene species were readily generated *in situ* by using a catalytic amount of triethylbenzylammonium chloride as the phase-transfer catalyst in the presence of aqueous NaOH and chloroform as described by Makosza and Wawrzyniewicz (28). By this method, methyl oleate and linoleate were transformed into the corresponding dichlorocyclopropane C_{18} derivatives **1** (78%) and **2** (93%), respectively.

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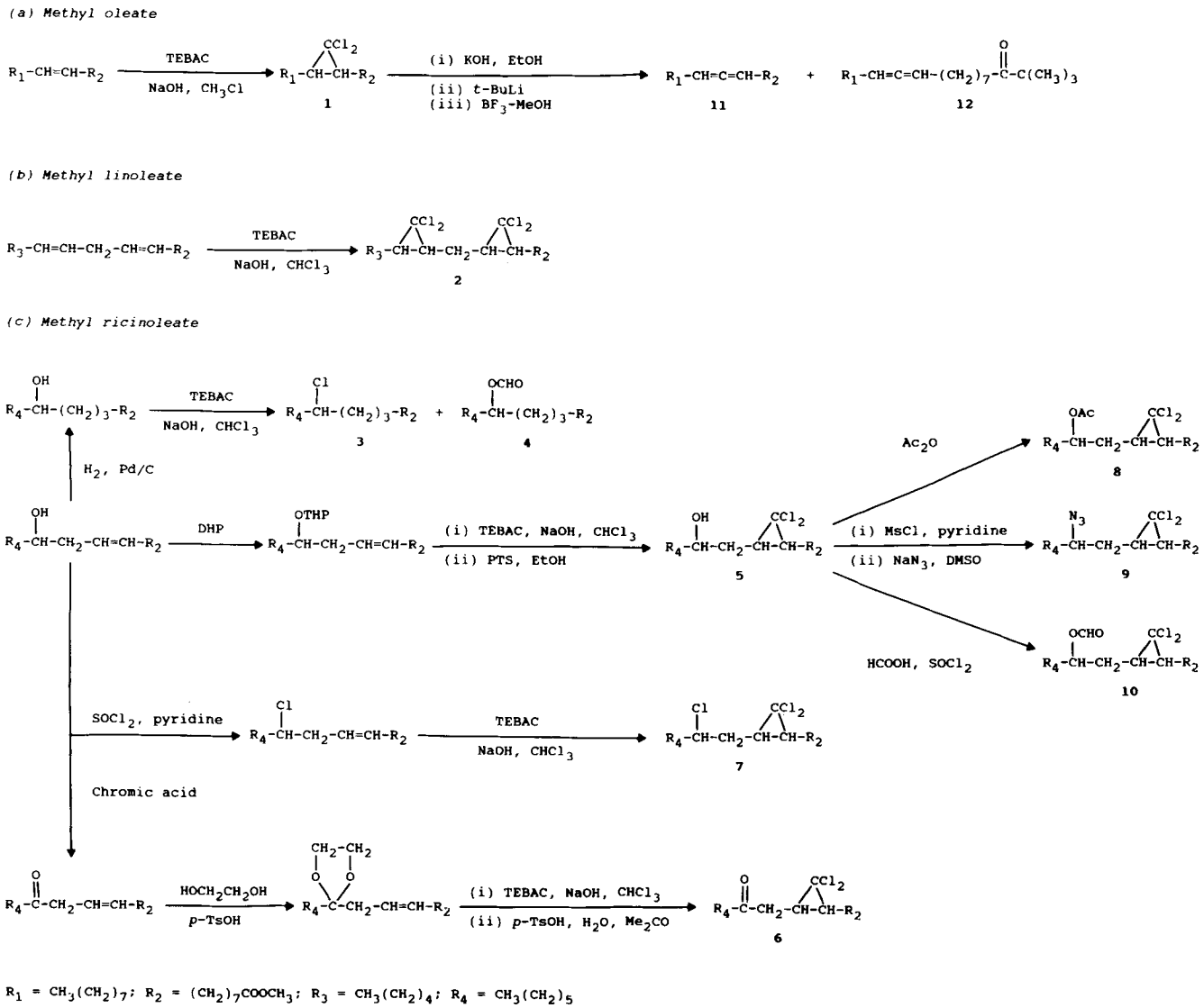
Abbreviations: ECL, equivalent chain length; GLC, gas-liquid chromatography; IR, infrared spectroscopy; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; Solvent A, petroleum ether (b.p. 40–60°C)/diethyl ether (95:5, v/v); Solvent B, petroleum ether (b.p. 40–60°C)/diethyl ether (4:1, v/v).



SCHEME 1

When methyl ricinoleate was reacted with dichlorocarbene, a complex mixture of products was obtained which could not be separated into individual components by silica gel thin-layer chromatography (TLC). The gas-liquid chromatographic (GLC) analysis of the crude product on SE-30 stationary phase showed the presence of at least three major components. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of the crude product showed an unexpected signal at δ 8.1 (*s*) and 160.8 ppm, respectively, suggesting the presence of an *O*-formyl function (-OCH=O). Methyl ricinoleate was hydrogenated to yield methyl 12-hydroxystearate, and the latter compound was reacted with dichlorocarbene generated from a mixture of triethylbenzylammonium chloride, aqueous NaOH and chloroform. Silica column chromatographic separation of the isolated product gave three fractions—methyl 12-chlorostearate (3, 49%); 12-*O*-formylstearate (4, 19%); and unreacted 12-hydroxystearate (32%). From these results it became apparent that the electrophilic dichlorocarbene species reacted with the hydroxyl group to give an alkoxydichloro carbanion intermediate which, upon hydrolysis, yielded the *O*-formyl C_{18} derivative 4. The same alkoxydichlorocarbene intermediate could also lose a chloride ion by α-elimination to form an alkoxymonochlorocarbene, which gave rise to the chloro C_{18} derivative (3) by elimination of a molecule of carbonmonoxide *via* and S_Ni reaction as described by Tabushi *et al.* (32). In order to prevent the attack of the hydroxy group in methyl ricinoleate by the dichlorocarbene species, the hydroxy group was protected by converting it to the *O*-tetrahydropyran-2′-yl (OTHP) derivative. Dichlorocyclopropanation by the phase-transfer method of the OTHP-protected methyl ricinoleate was readily accomplished. Removal of the protecting group gave the desired dichlorocyclopropane-hydroxy C_{18} derivative 5 (83%) as an oil.

Since carbonyl functions are also susceptible to reaction with dichlorocarbene species (33), the keto group of

methyl 12-oxo-*cis*-9-octadecenoate was converted to the 1,3-dioxolane derivative prior to treatment with dichlorocarbene. The desired dichlorocyclopropane-keto C_{18} derivative **6** (76%) was subsequently obtained after removal of the protecting group of the keto function.

Treatment of methyl ricinoleate with thionyl chloride gave methyl 12-chloro-*cis*-9-octadecenoate, which reacted with dichlorocarbene to give the corresponding 12-chloro-dichloro-cyclopropane C_{18} derivatives (**7**, 95%). Further transformation of the hydroxy function of compound **5** furnished the corresponding *O*-acetyl- (**8**, 98%), azido- (**9**, 33%) and *O*-formyl- (**10**, 27%) dichlorocyclopropane derivatives, demonstrating the stability of the dichlorocyclopropane ring towards the various reagents used in these conversions.

When the free acid of compound **1** was treated with *t*-butyl lithium, the chlorine atoms of the dichlorocyclopropane system were eliminated to yield a labile carbene intermediate (cyclopropylidene), which ring-opened to give after esterification a C_{19} allenic ester (**11**, 82%). A small amount of 2,2-dimethyl-3-oxo-11,12-heneicosadiene (**12**, 18%) was also obtained as a result of an attack of the *t*-butyl anion on the carboxylic acid function of the substrate. However, reaction of the free acids of the remaining dichlorocyclopropane derivatives (**2**, **5–10**) with *t*-butyl lithium failed to yield the corresponding allenic derivatives.

The dichlorocyclopropane derivatives (**2**, **5–10**) were all found to be stable during GLC analysis with the dichlorocyclopropane system contributing *ca.* 3.1 carbon numbers to the equivalent chain length (ECL) value of the parent fatty ester on SE-30 stationary phase. The chemical shift of the methine protons of the dichlorocyclopropane ring appeared as a multiplet at δ 1.6–1.8, due to the effects of the two chlorine atoms. In the ^{13}C NMR spectrum of compound **1**, the chemical shifts of the carbon atoms of the dichlorocyclopropane ring appeared at 33.0 ppm (C-9, C-10) and at 66.0 ppm for the chlorine substituted quaternary carbon atom. The shielding effect of the dichlorocyclopropane system on the two adjacent methylene carbons caused these nuclei to shift to 24.8 ppm (C-8, C-11). In the case of the *bis*-dichlorocyclopropane derivative (**2**), the ^{13}C NMR spectrum showed two distinct signals at 65.0 and 65.1 ppm for the quaternary carbon nuclei of the dichlorocyclopropane ring. Difficulties remain in the absolute assignment of these two signals, but the signal at 65.0 ppm seems to be due to the quaternary carbon of the dichlorocyclopropane ring nearer to the terminal methyl group. The effect of the hydroxy group at C-12 in compound **5** caused the methine carbon of the ring at C-10 to be shifted upfield to 29.7 ppm, while the methine carbon at C-9 appeared at 32.8 ppm. The presence of the hydroxy group was also confirmed by the signal at 70.9 ppm for the methine carbon atom (C-12) attached to the hydroxy group. In compound **6** the ring methine carbon atoms appeared at 32.7 and 27.9 ppm for C-9 and C-10, respectively, while the shift of the keto carbon atom appeared at 207.8 ppm. Compound **7** was characterized by the deshielding effect of the chlorine atom on the attached carbon atom (C-12), which appeared at 62.8 ppm. The carbon shifts of the methyl and carbonyl carbon of the *O*-acetyl group in compound **8** appeared at 21.1 and 170.4 ppm, respectively. The *O*-acetyl function was also characterized in the ^1H NMR spectrum by the signal at δ 2.09(*s*,3H) for the methyl protons of the *O*-acetyl function and by the multiplet at δ 5.0 for the methine proton at C-12. In compound **9** the azido group gave a characteristic strong absorption band at 2100 cm^{-1} (N_3, stretching) in the infrared spectrum, and the ^1H NMR shift of the methine proton at C-12 appeared at δ 3.4(*m*). The effect of the azido group on the chemical shift of the adjacent carbon atoms was rather similar to that observed for the chlorine atom in compound **7**. The *O*-formyl group in compound **10** was characterized by the signal appearing at δ 5.1(*m*) for the methine proton of C-12 in the ^1H NMR spectrum. In the ^{13}C NMR spectral analysis, the shift of the C-12 carbon atom of compound **10** appeared at 73.4 ppm.

EXPERIMENTAL PROCEDURES

Methyl oleate, methyl linoleate, triethylbenzylammonium chloride, *p*-toluenesulfonic acid, *t*-butyl lithium and methanesulfonyl chloride were obtained from Aldrich Chemical Company (Milwaukee, WI). Pyridinium *p*-toluenesulfonate was prepared by reaction of *p*-toluenesulfonic acid and pyridine (34); methyl ricinoleate was isolated from castor oil and methyl 12-oxo-*cis*-9-octadecenoate was prepared from methyl ricinoleate by oxidation as previously described (35). Methyl 12-hydroxystearate was obtained by hydrogenation of methyl ricinoleate over palladium on charcoal in MeOH. GLC analyses were carried out on a Hewlett-Packard (Hewlett-Packard Inc., Palo Alto, CA) model 5970 gas chromatograph fitted with a 10 m × 0.53 mm SE-30 microbore column using nitrogen (20 mL/min) as the carrier gas under isothermal conditions (220°C) and a flame ionization detector. External methyl ester standards (12:0, 14:0, 16:0 and 18:0) were used as reference compounds, and the equivalent chain length (ECL) values were calculated accordingly for each isomer (36). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL (JEOL Ltd., Tokyo, Japan) model FX90Q (90 MHz) pulse Fourier transform spectrometer operating at 90 and 22.5 MHz, respectively. Samples were dissolved in $CDCl_3$, which served as both the internal lock and secondary reference.

Dichlorocyclopropanation of methyl oleate. A mixture of methyl oleate (0.2 g, 0.68 mmol), chloroform (1.4 mL), triethylbenzylammonium chloride (30 mg) and aqueous NaOH (50%, 1 mL) was stirred at room temperature for 12 hr. Conc. HCl (5 mL) was added and the reaction mixture was extracted with chloroform (2 × 10 mL). The organic extract was washed with water (10 mL) and dried (Na_2SO_4). The organic solvent was evaporated and the residue separated by silica gel chromatography using Solvent A as eluent. Evaporation of the solvent gave the dichlorocyclopropane C_{18} derivative **1** (0.21 g, 78%) as an oil (27). TLC, R_f 0.6 (Solvent B). GLC, ECL 21.8 (SE-30). IR (cm^{-1}): 1740 (s), 1440(m), 805(m), 780(m). ^1H NMR (δ): 0.9(*t*,3H), 1.2–1.6(*m*,26H), 1.6–1.8(*m*,2H, C*H* ring), 2.3(*t*,2H), 3.6(*s*,3H). ^{13}C NMR (ppm): C-1, 174.2; C-2, 34.1; C-3, 25.0; C-4/C-6, 29.1–29.5; C-7, 28.8; C-8, 24.8; C-9, C-10, 33.0; C-11, 24.8; C-12, 28.7; C-13/C-15, 29.1–29.5; C-16, 31.9; C-17, 22.7; C-18, 14.1; $COOCH_3$, 51.4; $>CCl_2$, 66.0.

Dichlorocyclopropanation of methyl linoleate. A mixture of methyl linoleate (0.2 g, 0.68 mmol), chloroform (3

mL), triethylbenzylammonium chloride (30 mg) and aqueous NaOH (50%, 2 mL) was stirred at room temperature for 12 hr. Isolation and silica gel column chromatography gave the *bis*-dichlorocyclopropane C_{18} derivative **2** (0.29 g, 93%) as an oil (26). TLC, R_f 0.6 (Solvent B). GLC, ECL 24.6 (SE-30). IR (cm^{-1}): 1740(s), 1460(m), 805(m), 790(m). ^1H NMR (δ): 0.9(*t*,3H), 1.2–1.6(*m*,22H), 1.6–1.8(*m*,4H), 2.3(*t*,2H), 3.6(*s*,3H). ^{13}C NMR (ppm): C-1, 174.2; C-2, 34.1; C-3, 24.9; C-4/C-6, 29.1–29.7; C-7, 28.6; C-8/C-14, 24.8; C-9, 33.1; C-10, C-12, 31.2; C-11, 20.7; C-13, 33.9; C-15, 28.4; C-16, 31.5; C-17, 22.5; C-18, 14.0; $COOCH_3$, 51.4; and $>CCl_2$ 65.0 and 65.1.

Reaction of methyl 12-hydroxystearate with dichlorocarbene. A mixture of methyl 12-hydroxystearate (2.5 g, 7.9 mmol), aqueous NaOH (50%, 20 mL), chloroform (30 mL) and triethylbenzylammonium chloride (70 mg) was stirred for 12 hr at room temperature. Conc. HCl (50 mL) was added and the reaction mixture was extracted with chloroform (2 × 30 mL). The organic extract was washed with water (2 × 20 mL) and dried (Na_2SO_4). The solvent was evaporated under reduced pressure to give a mixture of crude products (2.3 g). Preparative TLC separation of 200 mg of the crude product using Solvent B as developer gave three fractions (A–C). The least polar fraction (A) consisted of methyl 12-chlorostearate, **3** (56 mg, 24%) as an oil. TLC, R_f 0.6 (Solvent B). GLC, ECL 19.9 (SE-30). ^1H NMR (δ): 0.9(*t*,3H), 1.2–1.6(*m*,24H), 1.6–1.8(*m*,4H), 2.3(*t*,2H), 3.6(*s*,3H), 4.0(*m*,1H, C*H*Cl). ^{13}C NMR (ppm): C-1, 174.2; C-2, 34.1; C-3, 24.8; C-4/C-9, 29.1–29.5; C-10, 26.5; C-11, 38.6; C-12, 64.3; (>*C*HCl); C-13, 38.6; C-14, 26.5; C-15, 29.3; C-16, 31.7; C-17, 22.6; C-18, 14.1; $COOCH_3$, 51.4. Fraction B gave methyl 12-*O*-formylstearate, **4** (22 mg, 9%) as an oil. TLC, R_f 0.45 (Solvent B). GLC, ECL 20.6 (SE-30). ^1H NMR (δ): 0.9(*t*,3H), 1.2–1.6(*m*,24H), 1.6–1.8(*m*,4H), 2.3(*t*,2H), 3.6(*s*,3H), 5.0(*m*,12-*H*), 8.1(*s*, -OC*H*=O). ^{13}C NMR (ppm): C-1, 174.2; C-2, 34.1; C-3, 25.0; C-4/C-9, 29.1–29.5; C-10, 25.2; C-11, 34.1; C-12, 74.6; C-13, 34.1; C-14, 25.2; C-15, 29.3; C-16, 31.7; C-17, 22.6; C-18, 14.1; $COOCH_3$, 51.4; -O*C*H=O, 161.1. Fraction C consisted of unreacted methyl 12-hydroxystearate (37 mg, 17%). TLC, R_f 0.2 (Solvent B). GLC, ECL 20.7 (SE-30). IR (cm^{-1}): 3400(s, br), 1740(s).

Dichlorocyclopropanation of OTHP derivative of methyl ricinoleate. A mixture of methyl ricinoleate (1.1 g, 3.6 mmol), 3,4-dihydro-2*H*-pyran (0.45 g, 5.4 mmol), pyridinium *p*-toluenesulfonate (0.1 g) and CH_2Cl_2 (7 mL) was stirred at room temperature for 12 hr. Diethyl ether (50 mL) was added and the ethereal mixture was washed with brine (2 × 10 mL) and dried (Na_2SO_4). Evaporation of the solvent gave the tetrahydropyran-2′-yloxy derivative of methyl ricinoleate (1.4 g). The latter compound was dissolved in chloroform (30 mL) and stirred with triethylbenzylammonium chloride (30 mg) and aqueous NaOH (50%, 8 mL) for 12 hr at 50°C. Dil. HCl (2M, 30 mL) was added and the reaction mixture was extracted with diethyl ether (2 × 30 mL). The extract was washed with water (20 mL) and dried (Na_2SO_4). The solvent was evaporated and the residue stirred with a solution of pyridinium *p*-toluenesulfonate (50 mg) in ethanol (20 mL) at 50°C for 4 hr. The solvent was evaporated under reduced pressure and the residue was extracted with diethyl ether (2 × 30 mL). The extract was washed with water (20 mL) and dried. Silica gel column chromatography gave the C_{18} dichlorocyclopropane-hydroxy C_{18} derivative **5** (1.6 g, 83%) as an oil. TLC, R_f 0.25 (Solvent B). GLC, ECL 24.3 (SE-30). ^1H NMR (δ): 0.9(*t*,3H), 1.2–1.6(*m*,20H), 1.6–1.8(*m*,6H), 2.3(*t*,2H), 2.6(*s*,1H, >CHO*H*), 3.65(*s*,3H), 3.8(*m*,1H, >C*H*OH). ^{13}C NMR (ppm): C-1, 174.3; C-2, 34.1; C-3, 24.9; C-4/C-6, 29.1–29.8; C-7, 28.5; C-8, 24.9; C-9, 32.8; C-10, 29.7; C-11, 32.6; C-12, 70.9, >*C*HOH; C-13, 37.7; C-14, 25.7; C-15, 29.3; C-16, 31.9; C-17, 22.6; C-18, 14.1, $COOCH_3$, 51.4; $>CCl_2$, 65.8.

Dichlorocyclopropanation of the 1,3-dioxolane derivative of methyl 12-oxo-cis-9-octadecenoate. A mixture of methyl 12-oxo-*cis*-9-octadecenoate (2 g, 6.5 mmol), ethylene glycol (18.5 mL), *p*-toluenesulfonic acid (0.1 g) and toluene (100 mL) was refluxed for 6 hr. The cooled reaction mixture was washed with water (50 mL) and dil. $NaHCO_3$ (10%, 30 mL) and dried (Na_2SO_4). The solvent was evaporated under reduced pressure. A mixture of chloroform (35 mL), triethylbenzylammonium chloride (0.1 g) and aqueous NaOH (50%, 30 mL) was added to the residue and stirred at 50°C for 1 hr and at room temperature for an additional 12 hr. Dil. HCl (2M, 100 mL) was added and the reaction mixture was extracted with chloroform (2 × 30 mL). The organic extract was washed with water (20 mL) and dried (Na_2SO_4). The solvent was evaporated under reduced pressure and the residue refluxed with *p*-toluenesulfonic acid (0.2 g), acetone (75 mL) and water (1 mL) for 1 hr. Water was added and the reaction mixture was extracted with petroleum ether (3 × 30 mL). The organic extract was washed with dil. $NaHCO_3$ (10%, 20 mL) and dried (Na_2SO_4). Silica column chromatographic separation gave dichlorocyclopropane-keto C_{18} derivative **6** (1.9 g, 76%) as an oil. TLC, R_f 0.5 (Solvent B). GLC, ECL 23.2 (SE-30). ^1H NMR (δ): 0.9(*t*,3H), 1.2–1.6(*m*,20H), 1.6–1.8(*m*,2H), 2.3(*t*,2H), 2.4(*t*,2H), 2.6(*t*,2H), 3.65(*s*,3H). ^{13}C NMR (ppm): C-1, 174.0; C-2, 34.1; C-3, 25.0; C-4/C-6, 28.5–29.3; C-7, 27.9; C-8, 25.2; C-9, 32.7; C-10, 27.9; C-11, 38.4; C-12, 207.8 (>C=O); C-13, 42.7; C-14, 23.9; C-15, 29.3; C-16, 31.8; C-17, 22.7; C-18, 14.2; $COOCH_3$, 51.4; $>CCl_2$, 65.2.

Dichlorocyclopropanation of methyl 12-chloro-cis-9-octadecenoate. A mixture of methyl ricinoleate (8.4 g, 28 mmol), thionyl chloride (4.3 g, 36 mmol) and pyridine (1 mL) was stirred at room temperature for 1 hr and refluxed for 30 min. Water (20 mL) was added and the reaction mixture was extracted with diethyl ether (3 × 30 mL). The organic extract was washed with dil. HCl (2M, 10 mL) and water (20 mL) and dried. Silica column chromatographic separation of the isolated product gave methyl 12-chloro-*cis*-9-octadecenoate (5.7 g, 62%) as an oil. A portion of the latter compound (0.2 g, 0.6 mmol) was stirred with chloroform (2 mL), triethylbenzylammonium chloride (20 mg) and aqueous NaOH (50%, 1.5 mL) at 50°C for 1 hr and at room temperature for an additional 12 hr. Dil. HCl (2M, 10 mL) was added and the reaction mixture was extracted with chloroform (2 × 15 mL). The organic extract was washed with water (10 mL) and dried. Silica column chromatographic purification of the product gave 12-chloro-dichlorocyclopropane C_{18} derivative **7** (0.24 g, 95%) as an oil. TLC, R_f 0.6 (Solvent B). GLC, ECL 23.3 (SE-30). ^1H NMR (δ): 0.9(*t*,3H), 1.2–1.6(*m*,20H), 1.6–1.8(*m*,6H), 2.3(*t*,2H), 3.7(*s*,3H), 3.9(*m*,1H). ^{13}C NMR (ppm): C-1, 174.2; C-2, 34.2; C-3, 25.0; C-4/C-6, 29.0–30.0; C-7, 28.8; C-8, 24.9; C-9, 33.0; C-10, 30.5; C-11, 34.1; C-12,

62.8; C-13, 38.7; C-14, 26.4; C-15, 29.5; C-16, 31.7; C-17, 22.6; C-18, 14.1; $COOCH_3$, 51.4; $>CCl_2$, 65.2.

Reaction of compound 5 with acetic anhydride. A mixture of compound **5** (0.16 g, 0.5 mmol) acetic anhydride (5 mL) and pyridine (1 mL) was stirred at room temperature for 12 hr. Water (10 mL) was added and the reaction mixture was extracted with petroleum ether (3 × 15 mL). The organic extract was washed with water (3 × 10 mL) and dried. Silica column chromatography of the isolated product gave the corresponding *O*-acetyl dichlorocyclopropane C_{18} derivative **8** (0.17 g, 98%). TLC, R_f 0.4 (Solvent B). GLC, ECL 23.7 (SE-30). ^1H NMR (δ): 0.9(*t*,3H), 1.2-1.6(*m*,20H), 1.6-1.8(*m*,6H), 2.09(*s*,3H), 2.3(*t*,2H), 3.7(*s*,3H), 5.0(*m*,1H). ^{13}C NMR (ppm): C-1, 173.9; C-2, 34.1; C-3, 25.0; C-4/C-6, 29.1-29.6; C-7, 28.5; C-8, 24.8; C-9, 32.6; C-10, 29.5; C-11, 29.4; C-12, 73.1; C-13, 33.9; C-14, 25.3; C-15, 29.3; C-16, 31.7; C-17, 22.5; C-18, 14.0; $COOCH_3$, 51.3; $>CCl_2$, 65.3; CH_3COO-, 170.4; CH_3COO-, 21.1.

Reaction of compound 5 with methanesulfonyl chloride followed by sodium azide. A mixture of compound **5** (0.3 g, 1.0 mmol), methanesulfonyl chloride (0.15 g, 1.3 mmol) and pyridine (10 mL) was stirred at 0-5°C for 2 hr. Cold dil. HCl (2M, 10 mL) was added and the reaction mixture was extracted with dichloromethane (2 × 30 mL). The organic extract was washed with dil. HCl (2M, 20 mL) and water (2 × 20 mL) and dried. The solvent was evaporated and the residue (0.29 g, 0.74 mmol) was stirred with sodium azide (50 mg, 0.77 mmol) in dimethylsulfoxide (10 mL) at 50°C for 4 hr. Water (50 mL) was added and the reaction mixture was extracted with diethyl ether (3 × 50 mL). The ethereal extract was washed with water (2 × 20 mL) and dried. Silica column chromatography of the isolated product gave azido-dichlorocyclopropane C_{18} derivative **9** (0.1 g, 33%) as an oil. TLC, R_f (Solvent B). GLC, ECL 23.8 (SE-30). IR (cm^{-1}): 2100 (s), 1740 (s). ^1H NMR (δ): 0.9(*t*,3H), 1.2-1.6(*m*,20H), 1.6-1.8(*m*,6H), 2.3(*t*,2H), 3.4(*m*,1H), 3.67(*s*,3H). ^{13}C NMR (ppm): C-1, 174.2; C-2, 34.1; C-3, 24.9; C-4/C-6, 29.0-29.5; C-7, 28.6; C-8, 25.1; C-9, 32.7; C-10, 29.6; C-11, 29.9; C-12, 62.1; C-13, 34.6; C-14, 26.1; C-15, 29.3; C-16, 31.7; C-17, 22.6; C-18, 14.1; $COOCH_3$, 51.4; $>CCl_2$, 65.4.

Reaction of compound 5 with formyl chloride. A mixture of formic acid (1.8 g, 39 mmol), pyridine (1.0 mL) and thionyl chloride (3.6 g, 30 mmol) was stirred at room temperature for 1 hr and heated at 80°C for an additional 1 hr. Compound **5** (0.3 g, 1.0 mmol) was added. The reaction mixture was stirred at 100°C for 1 hr and was left at room temperature for an additional 12 hr. Dil. HCl (2M, 20 mL) was added and the reaction mixture was extracted with diethyl ether (2 × 30 mL). The extract was washed with water (20 mL) and dried. Silica gel preparative TLC (Solvent B) gave *O*-formyl dichlorocyclopropane C_{18} derivative, **10** (70 mg, 27%), as an oil. TLC, R_f 0.45 (Solvent B). GLC, ECL 24.0 (SE-30). ^1H NMR (δ): 0.9(*t*,3H), 1.2-1.6(*m*,20H), 1.6-1.8(*m*,6H), 2.3(*t*,2H), 3.65(*s*,3H), 5.1(*m*,1H), 8.1(*s*,1H). ^{13}C NMR (ppm): C-1, 174.2; C-2, 34.1; C-3, 25.0; C-4/C-6, 29.0-29.7; C-7, 28.5; C-8, 24.9; C-9, 32.8; C-10, 28.5; C-11, 29.5; C-12, 73.4; C-13, 33.9; C-14, 25.2; C-15, 29.3; C-16, 31.7; C-17, 22.6; C-18, 14.1; $COOCH_3$, 51.4; $>CCl_2$, 65.4; -OCH=O, 160.8.

Reaction of free acid of compound 1 with t-butyl lithium. A mixture of the free acid of compound **1** (0.23 g, 0.63 mmol) and anhydrous diethyl ether (10 mL) was cooled to −10°C under nitrogen. *t*-Butyl lithium (1.7M in pentane, 2 mL) was added and the reaction mixture stirred for 1 hr at room temperature. Dil. HCl (2M, 15 mL) was added and the reaction mixture was extracted with diethyl ether (2 × 20 mL). The ethereal extract was washed with water (20 mL) and dried. The solvent was evaporated and the residue refluxed with BF_3/methanol complex (15%, w/w, 5 mL) and methanol (10 mL) for 15 min. Water (20 mL) was added and the reaction mixture was extracted with petroleum ether (2 × 20 mL). The organic extract was washed with water (10 mL) and dried. Silica gel chromatographic separation of the isolated product gave two main fractions. The more polar fraction (R_f 0.5; Solvent B) consisted of methyl 9,10-nonadecadienoate, **11** (81 mg, 82%) as an oil. GLC, ECL 19.1 (SE-30). IR (cm^{-1}): 1960 (w, C=C=C), 1740 (s, C=O). ^1H NMR (δ): 0.9(*t*,3H), 1.2-1.6(*m*,22H), 1.9(*m*, CH_2CH=C=$CHCH_2$, 4H), 2.3(*t*,2H), 3.66(*s*,3H), 5.1(*m*,2H, *CH*=C=*CH*). ^{13}C NMR (ppm): C-1, 174.3; C-2, 34.1; C-3, 25.0; C-4/C-8, 28.9-29.5; C-9, C-11, 90.8, 91.0; C-10, 203.8; C-12/C-16, 28.9-29.5; C-17, 31.9; C-18, 22.7; C-19, 14.1; $COOCH_3$, 51.4.

The less polar fraction (R_f 0.7; Solvent B) furnished 2,2-dimethyl-3-oxo-11,12-heneicosadiene **12** (18 mg, 18%). GLC, ECL 21.1 (SE-30). IR (cm^{-1}): 1960 (w, C=C=C), 1700 (s,C=O), 1460(s). ^1H NMR (δ): 0.9(*t*,3H), 1.1(*s*,9H, CH_3 of *t*-butyl), 1.2-1.6(*m*,22H), 1.9(*m*, CH_2HC=C=$CHCH_2$, 4H), 2.3(*t*,2H, CH_2C=O), 5.1(*m*,2H, *CH*=C=*CH*). ^{13}C NMR (ppm): C-1, C-2′, 26.4 (CH_3, *t*-butyl, 3C); C-2, 44.1; C-3, 216.1; C-4, 36.4; C-5, 24.0; C-6/C-10, 29.0-29.5; C-11, C-13, 90.0, 91.0; C-12, 203.8; C-14/C-18, 29.0-29.5; C-19, 31.9; C-20, 22.7; C-21, 14.1.

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METHOD

The Quantitation of Long-Chain Acyl-CoA in Mammalian Tissue

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Intracellular long-chain acyl-CoA esters are key metabolites in lipid metabolism. A rapid procedure was developed for the isolation of long-chain acyl-CoA from mammalian tissues. Acyl-CoA was extracted from the tissue with chloroform/methanol and separated from other lipid-containing metabolites by phase partition with solvents. The content and the molecular species of acyl-CoA were determined by gas-liquid chromatography. In rat liver and hamster heart, the total acyl-CoA content was estimated to be 83 ± 11 and 61 ± 9 nmol/g wet weight, respectively. The results obtained are comparable to those reported in previous studies. The relative ease of this procedure would permit the determination of acyl-CoA contents in a large number of samples.
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Acyl-coenzyme A (CoA) esters are intermediates involved in the acylation of lipids and proteins. The intracellular acyl-CoA content and composition have been shown to be important factors in determining the acyl group composition of phospholipids produced by reacylation (1–3). Acyl-CoA esters also act as modulators in the regulation of several enzyme systems (4,5) including the cardiac sodium pump (6) and protein kinase C (7). The identification of acyl-CoA as a metabolic regulator has led to the development of several assays for its quantitation (8). In general, the tissue is homogenized and proteins are precipitated from the homogenate. Subsequently, acyl-CoA is purified from the protein-free extract by column chromatography. Enzymatic or chemical analysis can then be employed for the quantitation of total acyl-CoA (9). The molecular species of the acyl-CoA can be determined by reverse-phase high-performance liquid chromatography (10,11). Since column chromatography is employed to purify acyl-CoA prior to its analysis, the procedure is not only time-consuming, but does not allow the simultaneous analysis of more than a few samples.

In the present study, a novel procedure was developed for the isolation of long-chain acyl-CoA in mammalian tissues. Acyl-CoA was extracted from tissue with chloroform/methanol and separated from other lipid-containing metabolites by phase partition. Subsequently, the total acyl-CoA content and the molecular species of acyl-CoA were determined by gas-liquid chromatography.

MATERIALS AND METHODS

[1-^{14}C]Oleoyl-CoA, [1-^{14}C]stearoyl-CoA, [1-^{14}C]palmitoyl-CoA, [1-^{14}C]arachidonoyl-CoA, [1-^{14}C]linoleoyl-CoA, [9,10(n)-^{3}H]palmitic acid, 1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine, 1-[^{14}C]palmitoyl carnitine, tri-[9,10-^{3}H]olein, and cholesteryl [1-^{14}C]oleate were obtained from Amersham Canada Limited (Oakville, Canada). The radiolabeled compounds were purified by solvent partition or thin-layer chromatography before use. Aqueous liquid scintillant (EcoLite) was purchased from ICN Biomedicals Canada Ltd. (St. Laurent, Canada). BF_3 methanol (12% w/w) was obtained through Supelco Canada Ltd. (Oakville, Canada). Heptadecanoic acid methyl ester was purchased from Sigma Chemical Company (St. Louis, MO). Thin-layer chromatographic plates (Sil-G25) were produced by Macherey-Nagel and purchased through Brinkmann Instruments (Rexdale, Canada). All other chemicals were of reagent grade and were obtained through Canlab Division of Travenol Canada Inc. (Mississauga, Canada). Theoretical aqueous phase (TAP), containing chloroform/methanol/water (3:48:47, v/v/v) and theoretical organic phase (TOP), containing chloroform/methanol/water (86:14:1, v/v/v) were prepared as described by Sheltawy and Dawson (12).

Mature male Sprague-Dawley rats, 250 ± 100 g, and Syrian Golden hamsters, 120 ± 20 g, were obtained from Charles River Can. Inc. (St. Constante, Canada). The animals were fed the rodent diet RMH 3000 (Agway Inc., Syracuse, NY) and tap water *ad libitum*. Animals were sacrificed by decapitation, and organs were rapidly removed and placed in liquid nitrogen. The frozen tissue (1 g) was placed in a test tube containing 10 mL of chloroform/methanol/water (1:1.5:0.2, v/v/v). Labeled acyl-CoA (0.1 μCi) or 100 nmol of acyl-CoA was added as an internal standard. The tissue was homogenized by a PT-30 polytron generator. In some experiments, 1 μCi of labeled phosphatidylcholine, palmitic acid, palmitoyl carnitine, cholesteryl oleate or triolein was added prior to homogenization.

The homogenate was centrifuged at 2,000 × *g* for 5 min. The supernatant was removed, and the pellet was extracted twice with 5 mL of chloroform/methanol/water (1:1.5:0.2, v/v/v). The supernatants were pooled and 1 mg of butylated hydroxytoluene was added to minimize the oxidation of the unsaturated long-chain acyl-CoA. Chloroform and water were added until the ratio of $CHCl_3/CH_3OH/H_2O$ was 4:2:3 (v/v/v). Phase separation of the mixture was achieved by centrifugation at 2,000 × *g*. The aqueous phase, containing long-chain acyl-CoA, was removed and the organic phase was extracted twice with 10 mL of TAP. The pooled aqueous phases were reduced to 3 mL by evaporation under reduced pressure.

The pooled aqueous phase was shaken with 6 mL chloroform and 0.75 mL saturated $(NH_4)_2SO_4$, and centrifuged at 2,000 × *g* for 10 min. The organic phase (containing long-chain acyl-CoA and some white precipitate) was removed and the aqueous phase was reextracted twice with 10 mL of TOP. The organic phases obtained from the washes were pooled and centrifuged at 2,000 × *g* for 5 min (to pellet the white precipitate). The supernatant was reduced under N_2, and long-chain acyl-CoA was extracted back into the aqueous phase by the addition of

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Abbreviations: BF_3, boron trifluoride; CoA, coenzyme A; DEGS, diethylene glycol succinate; TAP, theoretical aqueous phase; TLC, thin-layer chromatography; TOP, theoretical organic phase.

5 mL of TAP. After centrifugation, the aqueous phase was removed and the organic phase was re-extracted two times with 5 mL TAP. Vigorous mixing was essential for high acyl-CoA recovery during the extraction.

Samples obtained after phase partition were applied to a thin-layer chromatographic plate (G-25). The plate was developed in *n*-butanol/acetic acid/water (4:1:2, v/v/v). The fraction containing long-chain acyl-CoA (R_f 0.43) was identified by comparison with acyl-CoA standards. The R_f for long-chain acyl-CoA ($>C_{12}$) is different from hexanoyl-CoA and octanoyl-CoA (0.20), fatty acids (0.85), acyl carnitine (0.52), phosphatidylcholine (0.52) and phosphatidylethanolamine (0.65). The acyl-CoA in the fraction was eluted from the silica gel with 10 mL of the developing solvent.

Acyl-CoA content in the sample was determined by gas-liquid chromatography. The acyl groups in the sample were converted into methyl esters in the presence of boron trifluoride (13). Briefly, the solvent in the sample was evaporated under a stream of nitrogen, and 0.5 mL of boron trifluoride (BF_3) MeOH reagent was added to the dried sample. Methylation of the sample was carried out in a screw-capped tube which was heated at 96°C for 5 min. Subsequent to heating, 0.5 mL water and 1.5 mL petroleum hydrocarbon were added to the reaction mixture. The methyl esters formed were recovered in the petroleum hydrocarbon phase; the efficiency of methylation was better than 95%. The conversion of the acyl group to methyl ester by BF_3 appeared to be more efficient than the conversion to an alcohol by sodium borohydride (14). The methyl esters were analyzed on a Shimadzu Mini-2 gas-liquid chromatograph (Tekscience; Oakville, Canada) equipped with 15% DEGS (on 80/100 Chromosorb W/AW) columns. Heptadecanoic acid methyl ester was used as a standard for quantitation. The data obtained were analyzed by a Shimadzu Chromatopac CR601 integrator (Tekscience).

TABLE 1

Recovery of Radioactivity in Samples After Phase Partition[a]

Labeled compound added	Recovery (%)
Palmitoyl-CoA (16:0)	40 ± 4
Stearoyl-CoA (18:0)	43 ± 9
Oleoyl-CoA (18:1)	40 ± 6
Arachidonoyl-CoA (20:4)	39 ± 8
Palmitic acid	<0.1
Phosphatidylcholine	<0.1
Palmitoyl carnitine	<0.1
Cholesteryl oleate	<0.1
Triolein	<0.1

[a]Liver samples were homogenized and extracted as outlined in Materials and Methods. Radiolabeled lipid (1 μCi) was added to the sample prior to homogenization. The % radioactivity recovered in the sample after solvent partition is shown.

TABLE 2

Long-Chain Acyl-CoA Content and Distribution of Species in Samples After Phase Partition[a]

Acyl-CoA species	Distribution (%)
Myristoyl-CoA (14:0)	3 ± 2
Palmitoyl-CoA (16:0)	20 ± 5
Palmitoleoyl-CoA (16:1)	3 ± 2
Stearoyl-CoA (18:0)	33 ± 3
Oleoyl-CoA (18:1)	14 ± 6
Linoleoyl-CoA (18:2)	12 ± 4
Linolenoyl-CoA (18:3)	4 ± 2
Arachidonoyl-CoA (20:4)	8 ± 4
Others	3 ± 1
Total long-chain acyl-CoA	83 ± 11 nmol/g wet weight

[a]Acyl-CoA was isolated from rat liver homogenate by solvent partition as described in Materials and Methods. The resultant methyl esters were separated and quantitated by gas-liquid chromatography.

RESULTS AND DISCUSSION

The long-chain acyl-CoA in rat liver extract was found to partition in chloroform/methanol/water (4:2:3, v/v/v). The distribution of acyl-CoA in the aqueous and organic phases was monitored with labeled stearoyl-CoA, oleoyl-CoA and arachidonoyl-CoA. In all cases, over 95% of the radioactivity was found in the aqueous phase and less than 2% was recovered in the organic phase. When ammonium sulfate was added to the aqueous phase, a substantial portion of the labeled acyl-CoA was found in the organic phase. A 30% ammonium sulfate saturation in the aqueous phase was optimal for partitioning labeled CoA (68 ± 8%) into the organic phase. Most (72 ± 6%) of the labeled acyl-CoA in the organic phase was extracted back into the aqueous phase in the absence of ammonium sulfate. The overall recovery of acyl-CoA was 39–43% (Table 1). The yield obtained from this study is similar to that reported by Prasad *et al.* (15) but substantially higher than the value described by Olbrich *et al.* (16). Based on the results of Table 1, a 40% recovery was used to calculate the total acyl-CoA content.

The ability to separate long-chain acyl-CoA from other acyl-containing species by phase partition was examined. Labeled phosphatidylcholine, palmitic acid, triolein, cholesteryl oleate or palmitoyl carnitine was added to the tissue homogenate. After phase partition, the sample was analyzed for radioactivity. Less than 0.1% of the original radioactivity added to the homogenate was detected in the lipid fractions (Table 1). The results suggests that the long-chain acyl-CoA sample obtained after phase partition was not significantly contaminated with other lipid moieties.

The quantitation of long-chain acyl-CoA in the sample after phase partition was carried out by gas-liquid chromatography. The tissue homogenate was divided into two aliquots. An internal standard (100 nmol of acyl-CoA) was added to one aliquot prior to the isolation of acyl-CoA by phase partition. Subsequently, the acyl-CoA esters in the sample were converted into methyl esters and quantitated by gas-liquid chromatography. The quantity of each methyl ester species was determined by comparison with the methyl heptadecanoate standard.

The yield of acyl-CoA calculated from the internal standard (100 nmol of acyl-CoA) was in complete agreement with the values obtained from the labeled acyl-CoA (Table 1). The total long-chain acyl-CoA content as well as the distribution of the acyl species in rat liver are shown in Table 2. Our data (83 ± 11 nmol/g wet weight) are similar to those obtained in previous studies (15,17,18). However,

TABLE 3

Long-Chain Acyl-CoA Content and Distribution of Species in Samples After Thin-Layer Chromatography[a]

Acyl-CoA species	Distribution (%)
Myristoyl-CoA (14:0)	3 ± 2
Palmitoyl-CoA (16:0)	16 ± 6
Palmitoleoyl-CoA (16:1)	5 ± 2
Stearoyl-CoA (18:0)	35 ± 5
Oleoyl-CoA (18:1)	14 ± 4
Linoleoyl-CoA (18:2)	12 ± 4
Linolenoyl-CoA (18:3)	3 ± 2
Arachidonoyl-CoA (20:4)	6 ± 4
Others	6 ± 3
Total long-chain acyl-CoA content	81 ± 9 nmol/g wet weight

[a]Acyl-CoA was isolated from rat liver homogenate by solvent partition, followed by thin-layer chromatography as described in Materials and Methods. The resultant methyl esters were separated and quantitated by gas-liquid chromatography.

the value of arachidonoyl-CoA obtained in this study is lower than that reported by Prasad *et al.* (15). One possible explanation for this may be a difference in the diet of the animals used in the two studies. The rodent diet used in the present study contained a 7% lipid supplement whereas the diet used in the other study was a sucrose-casein based diet. It was reported by Masuzawa *et al.* (19) that the arachidonoyl-CoA level in the rat liver was significantly reduced (from 32% to 11%) by a diet supplemented with 10% soybean oil.

The requirement for further purification of the long-chain acyl-CoA after phase partition was also investigated. An aliquot of the acyl-CoA preparation obtained after phase partition was subjected to thin-layer chromatography with *n*-butanol/acetic acid/water (4:1:2, v/v/v) as solvent. Analysis of the chromatogram revealed that the R_f of acyl-CoA was different from the R_f values of free fatty acids, diacylglycerol, triacylglycerol and acyl carnitine (see Materials and Methods). The content and the composition of the acyl-CoA fraction obtained by thin-layer chromatography was analyzed by gas-liquid chromatography. As depicted in Table 3, the results obtained are similar to those found in the samples after phase partition (Table 2). Hence, the phase partition procedure alone appears to be adequate for the quantitation of acyl-CoA in a tissue extract.

In conclusion, phase partition is a rapid procedure for the determination of long-chain acyl-CoA content and composition in mammalian tissue. Using the same procedure, the analysis of long-chain acyl-CoA levels in the hamster heart was found to be 61 ± 9 nmol/g wet weight. The value obtained is similar to that reported for rat heart (19). The relative ease of the procedure would permit the determination of acyl-CoA content in a large number of samples. Analysis of long-chain acyl-CoA in smaller samples (biopsy samples) may also be feasible by increasing the sensitivity in gas-liquid chromatography.

ACKNOWLEDGMENT

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Measurement of Bile Acid Synthesis in Man by Release of $^{14}CO_2$ from [26-^{14}C]Cholesterol: Comparison to Isotope Dilution and Assessment of Optimum Reference Cholesterol Specific Activity

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Bile acid synthesis can be measured as release of $^{14}CO_2$ from [26-^{14}C]cholesterol divided by cholesterol specific activity, but this method has not been validated in human subjects. We made twelve comparisons of this CO_2 method to standard isotope dilution in six normal subjects and found a mean discrepancy of 6%. Linear regression analysis of one value with respect to the other revealed a correlation coefficient of 0.83 ($P < 0.001$), a Y-intercept close to zero (−4.98) and a slope close to 1 (1.06), suggesting good correspondence between the two methods. To assess the potential for error arising from use of serum cholesterol to estimate specific activity of cholesterol used for bile acid synthesis, we compared synthesis measured using serum free cholesterol specific activity to that measured using bile cholesterol specific activity, which is known to be near isotopic equilibrium with the precursor pool used for bile acid synthesis. Synthesis calculated in these two ways differed by less than 10%. The data indicate that the CO_2 method using either serum or bile cholesterol specific activity provides a valid estimate of bile acid synthesis in man.
Lipids 27, 68–71 (1992).

Bile acid synthesis is an important component of overall cholesterol homeostasis. In human subjects, bile acid synthesis is usually measured by Lindstedt isotope dilution kinetics (1) or fecal acidic sterol output (2). Both techniques are difficult and cannot be used to assess short-term changes in synthesis because sampling must be performed over periods of 4–10 days.

An alternative method, which enables measurement of short-term changes, is to quantitate synthesis by release of $^{14}CO_2$ from [26-^{14}C]cholesterol (3–5). During conversion of cholesterol to bile acid, carbon-26 is removed and quantitatively converted to CO_2. Output of $^{14}CO_2$ can be easily and accurately determined by quantitative collection of breath CO_2. Output of $^{14}CO_2$ is converted to bile acid production by dividing by specific activity of the cholesterol precursor pool used for bile acid synthesis.

Because this precursor pool specific activity is difficult to determine, it is usually approximated by substituting the specific activity of serum cholesterol (3–5). However, at least in certain circumstances, there is preferential use of newly synthesized cholesterol for bile acid synthesis (6). In that case, the specific activity of the cholesterol precursor pool used for bile acid synthesis would be lower than the specific activity of the serum cholesterol, and the calculated bile acid synthesis rate would be falsely low. An approach that might theoretically avoid this potential problem would be to use biliary cholesterol specific activity for calculation of bile acid synthesis because the pool of cholesterol used for bile acid synthesis appears to be in near equilibrium with the pool of cholesterol destined for secretion into bile (6).

In the present study we have assessed the accuracy of this CO_2 method in human volunteers by comparing it to the isotope dilution technique of Lindstedt. In addition, we have evaluated the possibility that biliary cholesterol specific activity might be superior to that of serum cholesterol for calculation of bile acid synthesis by the CO_2 method.

MATERIALS AND METHODS

Six male volunteers were studied. All had participated in two earlier studies of effects of lovastatin on biliary lipid secretion and bile acid synthesis (7,8). As part of one of these studies (7), each had had bile acid synthesis measured by isotope dilution, as originally described by Lindstedt (1), in two separate periods—once in a control period and once after ingesting lovastatin 40 mg b.i.d. for 5–6 weeks. As part of the other study (8), each volunteer had bile acid synthesis measured by the CO_2 method in the same two periods. This measurement was performed one week after the isotope dilution measurement.

The volunteers ranged in age from 51–68 years, in percent ideal body weight from 98–132%, and in serum cholesterol from 140–210 mg/dL. Two of the volunteers had hypertriglyceridemia (412 and 478 mg/dL) without hypercholesterolemia. Their results did not appear to differ from those of the other four subjects.

Informed consent was obtained from all volunteers prior to study. All study protocols were approved by the Subcommittee on the Use of Human Subjects in Research of the Minneapolis VA Medical Center.

Bile acid synthesis was measured as release of $^{14}CO_2$ from administered [26-^{14}C]cholesterol (New England Nuclear, Boston, MA) as described previously (5). Briefly, the subject was given 20–50 μCi of [26-^{14}C]cholesterol at least one week prior to study. Expired CO_2 was quantitatively collected by having the subject place his head in a plexiglass hood. Air from this hood was drawn rapidly through a series of collection traps containing a solution of phenethylamine to trap CO_2 and Liquifluor (New England Nuclear) to permit scintillation counting. Duplicate 8–10 min collections were done 30 min apart. All collections were performed at the same time of day, between 12 p.m. and 1:00 p.m., to avoid changes resulting from circadian variation. Bile acid synthesis was calculated by dividing output of $^{14}CO_2$ by specific activity of cholesterol. Unless otherwise stated, biliary cholesterol specific activity was used in this calculation.

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Specific activities of both biliary and serum free cholesterol were measured by gas-liquid chromatography and liquid scintillation counting (5,9). For analysis of serum samples, free cholesterol was first isolated from a petroleum hydrocarbon (b.p. 30–60°C) extract by thin-layer chromatography using a solvent system of diethyl ether/heptane (55:45, v/v). Biliary cholesterol for measurement of specific activity was obtained during measurement of stimulated secretion rates of biliary lipids by the method of Grundy and Metzger (10).

RESULTS

Bile acid synthesis measured by isotope dilution averaged 105 ± 11% (mean ± SEM) of synthesis measured by the CO_2 method. Each of the individual measurements of these two variables is plotted in Figure 1, together with the regression line. Correlation coefficient for this regression was 0.83 ($P < 0.001$). The Y-intercept of the regression was close to zero (−4.98) and the slope was close to 1 (1.06), suggesting good correspondence between synthesis rates measured by the two methods over a fairly wide range of synthesis rates.

Bile acid synthesis by the CO_2 method calculated using the serum free cholesterol specific activity averaged 91 ± 4% of synthesis calculated using the bile cholesterol specific activity. The tendency for synthesis calculated using the serum cholesterol specific activity to be slightly lower than synthesis calculated using the bile cholesterol specific activity reflects the slightly lower specific activity of bile as compared to the serum free cholesterol reported earlier (8). As shown in Figure 2, however, the points clustered close to the line of identity suggesting little difference in values calculated by the two methods.

DISCUSSION

Measurement of bile acid synthesis as release of $^{14}CO_2$ from [26-^{14}C]cholesterol is considerably easier and quicker than measurement by isotope dilution or fecal acidic sterol balance. Until now, however, the method has not been validated in human subjects. Redinger *et al.* (4) compared this CO_2 method to fecal acidic sterol output in baboons and found the methods provided approximately equal values for bile acid synthesis in animals with an intact enterohepatic circulation.

In the present study we found reasonably good correspondence of bile acid synthesis measured by the CO_2 method as compared to standard isotope dilution (Fig. 1). The correspondence was not perfect, presumably in part because of the complexity of sampling and analysis, especially in the isotope dilution method. However, the observed average discrepancy of 6% between the two methods is small compared to the magnitude of changes in bile acid synthesis one is usually attempting to assess. Moreover, the apparent linear relationship between values returned by the two methods with a linear regression Y-intercept near zero and a slope near 1 suggests that synthesis rates over the range found in our subjects were measured equally well by either method. Thus the present study confirms the findings in baboons (4) and provides the first validation of this technique in human subjects.

Previous studies from our laboratory and others indicate that in normolipidemic subjects, isotope dilution and fecal acidic sterol balance yield comparable values for bile acid synthesis (11,12). However, there is some evidence that in hypertriglyceridemic subjects isotope dilution returns a higher rate of bile acid synthesis than fecal acidic sterol output (12). Two of our subjects had elevated triglycerides. In one of them synthesis by isotope, dilution was about 10% higher than by the CO_2 method in both control and lovastatin periods. In the other synthesis by isotope, dilution was actually lower than by the CO_2 method in both periods. Thus, these limited data do not suggest that isotope dilution overestimates bile acid synthesis compared to the CO_2 method in hypertriglyceridemic subjects.

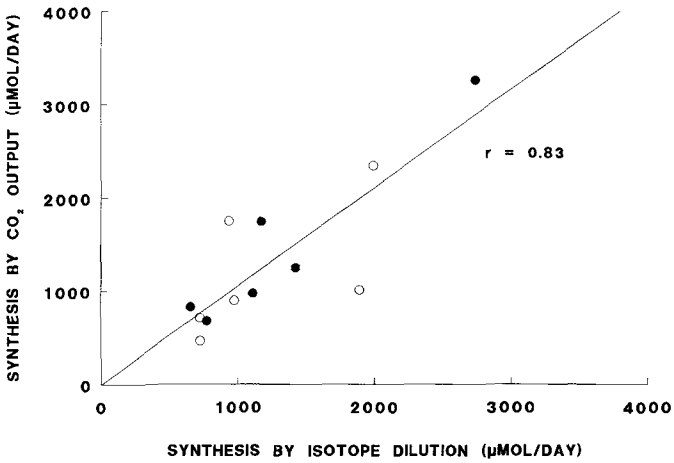

FIG. 1. Relationship between bile acid synthesis determined by isotope dilution *vs.* release of $^{14}CO_2$ from [26-^{14}C]cholesterol. The two methods correlated reasonably well ($r = 0.83$, $P < 0.001$) over about a four-fold range of synthesis rates. Moreover, the Y-intercept was close to zero (−4.98) and the slope was close to 1 (1.06), suggesting that there was no systematic bias of one method as compared to the other. Open circles represent the period of lovastatin ingestion, while closed circles represent the control period off lovastatin.

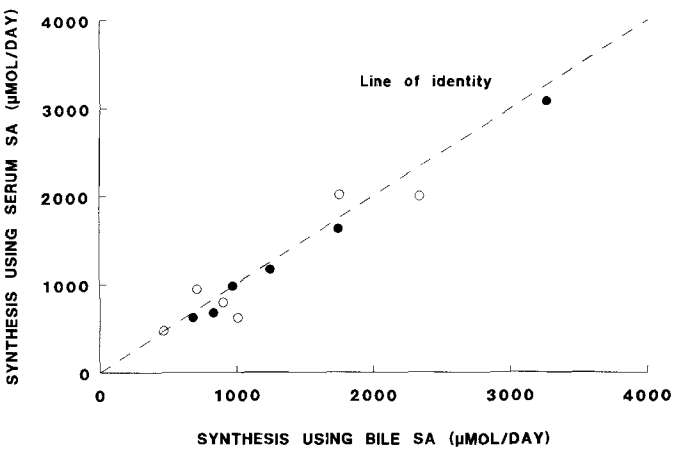

FIG. 2. Relationship of bile acid synthesis determined by the CO_2 method calculated with the biliary cholesterol specific activity *vs.* that calculated with the serum unesterified cholesterol specific activity. The two specific activities provided nearly identical values for bile acid synthesis, although there was a slight tendency for synthesis values calculated with the serum specific activity to be lower. As in Figure 1, open circles represent the lovastatin period and closed circles represent the control period.

We have previously reported that bile acid synthesis measured by the CO_2 method undergoes circadian variation (5). Therefore, in comparing this method to isotope dilution, which provides an average value of synthesis over four days, measurements of $^{14}CO_2$ output must be done at an appropriate time of day. We selected midday for determination of $^{14}CO_2$ output because our previous data suggest that this is the time most likely to correspond to mean daily synthesis (5). Nevertheless, in any individual, synthesis might be somewhat more or less than average at this time of day, which represents a potential for some variation and inaccuracy when using the CO_2 method to estimate mean daily synthesis.

It is also important in calculating bile acid synthesis by the CO_2 method to use the best possible estimate of cholesterol specific activity in the precursor pool destined for bile acid synthesis. Because cholesterol synthesis and bile acid synthesis take place in proximity to one another, newly synthesized cholesterol is likely to be disproportionately represented in this precursor pool. If so, the serum free cholesterol specific activity would be somewhat higher than that of the precursor pool and would yield a falsely low estimate of bile acid synthesis. A remedy for this potential problem is suggested by the observation that the precursor pool destined for bile acid synthesis and that destined for secretion into bile are near equilibrium (6). We therefore examined the possibility that the specific activity of biliary cholesterol might provide a better estimate than the serum cholesterol of the specific activity of the precursor pool used for bile acid synthesis. As reported earlier (8), serum free cholesterol specific activity did tend to be higher than that of biliary cholesterol, but the difference was less than 10%. Consequently, as shown in Figure 2, both the serum free cholesterol and the biliary cholesterol specific activity yielded reasonably accurate calculated values for bile acid synthesis.

In summary, these studies indicate that in human subjects with an intact enterohepatic circulation, the $^{14}CO_2$ technique provides a reasonably accurate measure of bile acid synthesis. Either serum or bile can be used to determine the reference cholesterol specific activity. The method has the advantages of easily and quickly providing a short-term measure of bile acid synthesis, but has the disadvantage of requiring administration of fairly large amounts of radioactive cholesterol (20–50 μCi). The method also provides no information about kinetics or pool sizes of bile acid.

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APPENDIX

For purposes of illustration, the following is an example of calculations performed on one subject.

Bile Acid synthesis by isotope dilution (Lindstedt method). This measurement was carried out as described originally by Lindstedt (1), and later by our own laboratory (9). The evening prior to sampling, the subject was given 1.357×10^7 DPM of [24-^{14}C]cholic acid and 1.196×10^7 DPM of [24-^{14}C]chenodeoxycholic acid. Each of the subsequent four mornings (days 0.5, 1.5, 2.5 and 3.5), a sample of gallbladder bile was obtained and the specific activity of these two bile acids was measured in duplicate.

For cholic acid these specific activities were—12,690, 7193, 3783, and 1715 DPM/μmole. Linear regression of the natural logarithm of specific activity (y) *vs.* time after isotope administration (x) was performed and provided a slope of 0.665 day^{-1} (which is the fractional turnover of cholic acid) and a Y-intercept of 9.8331 (which is the natural logarithm of the specific activity at time zero). The antilog of the Y-intercept provided the specific activity at time zero:

$$\text{specific activity} = \exp(9.8331) = 18{,}640 \text{ DPM}/\mu\text{mole}$$

Dividing the specific activity at time zero into the administered amount of cholic acid administered yielded cholic acid pool size:

$$\text{pool} = (1.357 \times 10^7)/18{,}640 = 728 \ \mu\text{mole}$$

Finally multiplying the pool size by the fractional turnover of cholic acid yielded synthesis rate of cholic acid:

$$\text{synthesis} = (0.665) \times (728) = 484 \ \mu\text{mole/day}$$

Similar analysis was done for chenodeoxycholic acid yielding specific activities of 18,100, 12,860, 7527, 4204 DPM/μmole. Linear regression then yielded a slope of 0.491 day^{-1} and a Y-intercept of 10.1169. Again, taking the antilog of the Y-intercept provided a specific activity at time zero:

$$\text{specific activity} = \exp(10.1169) = 24{,}758 \text{ DPM}/\mu\text{mole}$$

Dividing that into the amount of labelled chenodeoxycholic acid administered yielded pool size of chenodeoxycholic acid:

$$\text{pool} = (1.196 \times 10^7)/24758 = 483 \ \mu\text{moles}$$

Multiplying this pool size by the fractional turnover of chenodeoxycholic acid yielded synthesis rate of chenodeoxycholic acid:

$$\text{synthesis} = (0.491) \times (483) = 237\ \mu\text{mole/day}$$

Finally, to obtain total bile acid synthesis by isotope dilution, synthesis rates for these two bile acids were summed:

$$\text{total synthesis} = 484 + 237 = 721\ \mu\text{mole/day}$$

Bile acid synthesis by CO_2 method. This measurement was carried out as previously described (5). Immediately following completion of four days of bile collection, the same subject was given about 25 μCi of [26-^{14}C]-cholesterol. One week later, we performed quantitative collections of breath $^{14}CO_2$ for three separate 8-min periods. In these 8-min collections, output of $^{14}CO_2$ averaged 2482 DPM. Multiplying by (1440/8) provided average daily output of $^{14}CO_2$ (= 446,760 DPM/day). On the same day, a sample of stimulated bile was obtained for measurement of cholesterol specific activity (= 630 DPM/μmole). Dividing daily output of $^{14}CO_2$ by this cholesterol specific activity yielded average daily bile acid synthesis rate:

$$\text{synthesis} = (446{,}760/(630) = 709\ \mu\text{mole/day}$$

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COMMUNICATION

Two Novel Naturally Occurring α-Methoxy Acids from the Phospholipids of Two Caribbean Sponges

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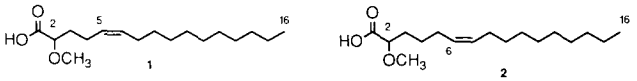
The novel (5Z)-2-methoxy-5-hexadecenoic acid (1) was identified in the phospholipids of the sponge *Tethya crypta* while the also novel acid (6Z)-2-methoxy-6-hexadecenoic acid (2) was found in the phospholipids of the Caribbean sponge *Spheciospongia cuspidifera*. The methoxy-fatty acids were mainly associated with phosphatidylethanolamine. The double bond positions were determined by gas chromatography/mass spectrometry on the corresponding dimethyldisulfide adducts and the double bond stereochemistry was ascertained by gas chromatography/Fourier transform infrared spectroscopy. The fatty acid composition of the two sponges is reported.
***Lipids* 27, 72-74 (1992).**

Phospholipids containing α-substituted fatty acids are quite rare in nature, but sponges have provided the most striking examples. Two particularly interesting examples have been the phospholipid-bound α-methoxy and α-hydroxy fatty acids. Ayanoglu *et al.* (1) reported that the African sponge *Higginsia tethyoides* contains α-methoxy acids, such as 2-methoxy-17-tetracosenoic acid (24:1n-7), 2-methoxy-19-hexacosenoic acid (26:1n-7), and 2-methoxy-21-octacosenoic acid (28:1n-7). We also recently encountered a series of phospholipids containing α-hydroxy fatty acids, namely 2-hydroxydocosanoic (22:0) and 2-hydroxytricosanoic (23:0) acids which were isolated from the sponge *Amphimedon compressa* (2). Ayanoglu *et al.* (3) have also described another class of demospongic acids, namely totally saturated 2-acetoxy C_{22}-C_{30} acids from the sponge *Polymastia gleneni*. These probably are the only examples of 2-oxo-substituted fatty acids in the phospholipids of sponges, mostly occurring in phosphatidylethanolamine.

We now wish to report the isolation and structure elucidation of the new phospholipid fatty acid (5Z)-2-methoxy-5-hexadecenoic acid (1) from the sponge *Tethya crypta*, and (6Z)-2-methoxy-6-hexadecenoic acid (2) which was found in the Caribbean sponge *Spheciospongia cuspidifera*.



SCHEME 1

EXPERIMENTAL PROCEDURES

T. crypta and *S. cuspidifera* were collected in July, 1989, near the shelf edge of La Parguera, Puerto Rico, at a depth of 15 m. The sponges (500 g) were washed in sea water, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction with 600 mL of chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids (100 mg) were separated by column chromatography on silica gel (60-200 mesh) using a procedure similar to that of Privett *et al.* (4). The phospholipid classes were fractionated by preparative thin-layer chromatography (TLC) using silica gel G and chloroform/methanol/water (25:10:1, v/v/v) as solvent. The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic hydrogen chloride (5) followed by column chromatographic purification and elution with hexane/diethyl ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatography/mass spectrometry using either a Hewlett-Packard 5995 A gas chromatograph/mass spectrometer (Hewlett-Packard, Palo Alto, CA) or a Hewlett-Packard 59970 MS ChemStation equipped with a 30 m × 0.32 mm nonpolar fused silica column (Supelco, Bellefonte, PA) with SPB™-1 as the bonded phase. Gas chromatography/Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet (Madison, WI) 740 FT-IR spectrometer. For the location of double bonds, dimethyldisulfide derivatives were prepared by dissolving the esters (2 mg) in dimethyldisulfide (0.2 mL) and adding a solution (0.05 mL) of iodine in diethyl ether (60 mg/mL). The solution was heated at 50°C for 24 hr followed by the standard purification method (6). Hydrogenations were carried out in 10 mL of absolute methanol in the presence of catalytic amounts of platinum oxide (PtO_2). Mass spectral data for the key fatty acids described are presented here:

Methyl (5Z)-2-methoxy-5-hexadecenoate. MS (70 eV) *m/z* (relative intensity), 298 (M^+,1), 266 (M^+-CH_3OH,12), 239 ($M^+-CO_2CH_3$,5), 207 (1.7), 206 (2.6), 180 (1), 150 (3), 139 (3), 135 (3), 127 (2), 123 (4), 121 (4), 117 (2), 111 (12), 109 (8), 104 (100), 97 (16), 95 (13), 93 (16), 89 (9), 87 (6), 83 (12), 81 (14), 79 (11), 75 (5), 71 (7), 69 (14), 67 (16), 57 (10), 55 (21).

*Methyl 2-methoxy-5,6-*bis*(methylthio)hexadecanoate.* (3) MS (70 eV) *m/z* (relative intensity), 392 (M^+,4), 201 (31), 191 (100), 160 (10), 159 (91), 131 (52), 111 (17), 97 (23), 95 (11), 87 (18), 85 (13), 83 (19), 81 (13), 79 (13), 75 (15), 74 (17), 71 (27), 69 (26), 67 (19), 61 (33), 59 (17), 57 (20), 55 (39).

Methyl (6Z)-2-methoxy-6-hexadecenoate. MS (70 eV) *m/z* (relative intensity), 298 (M^+,1), 266 (M^+-CH_3OH,4), 239 ($M^+-CO_2CH_3$,20), 207 (6), 206 (7), 180 (6), 150 (11), 139 (5), 138 (8), 136 (10), 127 (8), 123 (13), 121 (12), 117 (6), 111 (18), 109 (19), 104 (100), 97 (21), 95 (39), 93 (17), 88 (12), 87 (16), 83 (18), 81 (35), 79 (19), 74 (16), 71 (30), 69 (25), 67 (37), 57 (22), 55 (48).

*Methyl 2-methoxy-6,7-*bis*(methylthio)hexadecanoate.* (4) MS (70 eV) *m/z* (relative intensity), 392 (M^+,3), 206 (9), 205 (100), 187 (37), 173 (37), 145 (23), 125 (12), 97 (32), 95 (16), 93 (11), 87 (64), 85 (12), 83 (28), 81 (21), 79 (12), 75 (11), 71 (45), 69 (27), 67 (29), 61 (44), 57 (19), 55 (46).

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Abbreviations: ECL, equivalent chain length; GC/MS, gas chromatography/mass spectrometry; TLC, thin-layer chromatography; FTIR, Fourier transform infrared spectroscopy.

TABLE 1

Principal Fatty Acids from *Tethya crypta* and *Spheciospongia cuspidifera*[a]

Fatty acid	Abundance (%)	
	T. crypta	*S. cuspidifera*
Tetradecanoic (14:0)	3	7
4,8,12-Trimethyltridecanoic (16:0)	2	11
Pentadecanoic (15:0)[b]	5	—
9-Hexadecenoic (16:1)	3	2
Hexadecanoic (16:0)[b]	16	10
Heptadecanoic (17:0)	2	3
Octadecenoic (18:1)[c]	2	5
2-Methyloctadecanoic (19:0)	5	—
2-Methoxy-5-hexadecenoic (16:1)	2	—
2-Methoxy-6-hexadecenoic (16:1)	—	4
Octadecanoic (18:0)	5	5
Nonadecanoic (19:0)[b]	4	6
Eicosanoic (20:0)	3	5
Heneicosanoic (21:0)	2	2
2-Hydroxydocosanoic (22:0)	4	2
2-Hydroxytricosanoic (23:0)	1	1
2-Hydroxytetracosanoic (24:0)	2	5
2-Hydroxypentacosanoic (25:0)	4	2
5,9-Pentacosadienoic (25:2)	6	4
5,9-Hexacosadienoic (26:2)	15	18

[a]Some minor acids are not included in this table.

[b]These normally were a mixture of the branched iso-anteiso acids.

[c]Includes the Δ9 and Δ11 isomers.

RESULTS AND DISCUSSION

Capillary gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) of the mixture gave the principal acids described in Table 1 for *T. crypta*, and *S. cuspidifera*. As can be seen from the Table, both *T. crypta* and *S. cuspidifera* possess a series of α-hydroxy fatty acids, the most abundant being those between C_{22}–C_{25} carbons long. *T. crypta* also has a rare 2-methyloctadecanoic acid, which tends to indicate the propensity of this sponge for phospholipids with α-substituted fatty acids. An unusual fatty acid methyl ester from *T. crypta* (2% abundance) was difficult to characterize due to the small amounts of this acid in the complex mixture of more than 25 fatty acid methyl esters. Capillary GC on a SPB™-1 30-m column and GC/MS became the principal tools for the characterization. The unknown ester exhibited unusual chromatographic properties, namely an equivalent chain length (ECL) of 17.20, which is unusual for the molecular weight of this methyl ester. The mass spectrum showed a molecular peak at *m/z* 298 (1%) and a base peak at *m/z* 104, arising from a McLafferty rearrangement (1). The additional 30 amu, when compared with the typical base peak of saturated fatty acid methyl esters at *m/z* 74, could only be accounted for by a methoxy group at the 2-position. Important for the characterization were also fragmentation ions at *m/z* 266 ($[M-MeOH]^+$, 2%), *m/z* 239 ($[M-COOCH_3]^+$, 5%), and *m/z* 207 ($[M-COOCH_3-CH_3OH]^+$, 2%). In fact, comparison of the mass spectrum with spectra for other 2-methoxy acids (1) confirmed that we have identified a novel 2-methoxyhexadecenoic acid. The double bond position was determined by preparing the corresponding dimethyldisulfide adduct, a convenient method for the location of double bonds by mass spectrometry (6,7). The mass spectrum of the corresponding dimethyldisulfide adduct afforded a molecular ion peak at *m/z* 392 (M^+, 4%). The double bond was readily determined to be at carbon 5 by the fragmentations at *m/z* 201 ($[C_{11}H_{22}SCH_3]^+$, 31%) and *m/z* 191 ($[C_8H_{15}SO_3]^+$, 100%) since this constitutes cleavage between carbons 5 and 6. There was also a prominent peak at *m/z* 159 (91%) corresponding to the *m/z* 191 fragment with the loss of methanol; these fragmentations are shown in 3 (Fig. 1). The experimental data thus support the structure of a (5Z)-2-methoxy-5-hexadecenoic acid (1) as the new compound. The capillary GC-FTIR spectrum showed hydrocarbon and ester bands at 3020, 2925, 2850 and 1743 cm^{-1}, respectively. There was no prominent absorption in the 980–968 cm^{-1} region, that would be indicative of *cis* rather than *trans* unsaturation.

The new methoxy fatty acid methyl ester (4% abundance) from *S. cuspidifera* also exhibited a long retention time in GC (ECL, 17.22), unusual for the molecular weight of this ester. The mass spectrum indicated a molecular weight of 298 (1%) and the same base peak at *m/z* 104 (McLafferty rearrangement). Other key fragmentations were also found at *m/z* 266 ($[M-MeOH]^+$, 6%), *m/z* 239 ($[M-COOCH_3]^+$, 5%) and *m/z* 207 ($[M-COOCH_3-CH_3OH]^+$, 6%. This suggested that the compound was identical to or an isomer of 1. The dimethyldisulfide adduct was again prepared and the mass spectrum unequivocally determined the double bond position to be at carbon 6. In this case, the molecular ion peak was also observed at *m/z* 392 (M^+, 3%) and the double bond was deduced from the prominent fragments at *m/z* 187 ($[C_{10}H_{20}SCH_3]^+$, 37%) and *m/z* 205 ($[C_9H_{17}SO_3]^+$, 100%), this constitutes cleavage between carbons 6 and 7 as exemplified in 4. There was also a prominent peak at *m/z* 173 (37%) corresponding to the 205 fragment with the

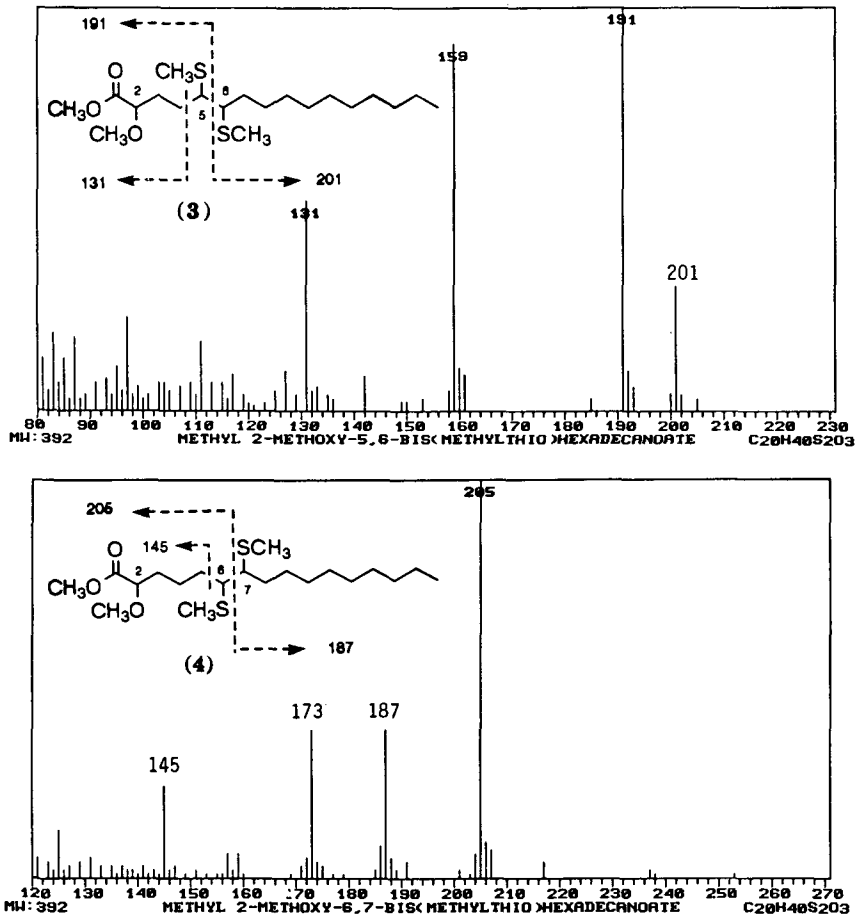

FIG. 1. Partial mass spectra of methyl 2-methoxy-5,6-*bis*(methylthio)hexadecanoate (3) and methyl 2-methoxy-6,7-*bis*(methylthio)hexadecanoate (4) displaying the key fragmentations used for the localization of the double bonds.

concomitant loss of methanol. The capillary GC-FTIR spectrum showed hydrocarbon and ester bands at 3020, 2925, 2850 and 1743 cm^{-1}, respectively. There was no prominent absorption in the 980-968 cm^{-1} region, indicating *cis* rather than *trans* unsaturation. The compound in question was thus (6Z)-2-methoxy-6-hexadecenoic acid (2) which is also unprecedented in nature.

The acids reported here were mainly associated with phosphatidylethanolamine. Naturally occurring 2-hydroxy fatty acids are known to possess the R configuration and the determination by Ayanoglu *et al.* (8) that methoxy fatty acids from sponges have the R configuration at the α-carbon tends to indicate that the compounds presented here also possess the R configuration. The biosynthetic origin of these novel acids is presently a matter of speculation, and much biosynthetic work is needed in order to put in perspective the origin of these novel α-methoxy acids.

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Occurrence of Wax Esters and 1-*O*-Alkyl-2,3-diacylglycerols in Goat Epididymal Sperm Plasma Membrane

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Two unusual lipid classes were detected by thin-layer chromatography in the neutral lipids derived from goat cauda-epididymal sperm plasma membrane. The lipids were identified as wax esters and 1-*O*-alkyl-2,3-diacylglycerols based on chromatographic properties, identity of their hydrolysis products, and infrared/^{1}H nuclear magnetic resonance spectral evidence. The membrane contained *ca.* 3 and 5 μg/mg protein of wax esters and alkyldiacylglycerols, respectively. The relative proportions of wax esters and alkyldiacylglycerols in the total neutral lipids were 1.5% and 2.4%, respectively. The lipids contained fatty acids with chain lengths of C_{14} to C_{22}. The major fatty acids of the wax esters were 14:0, 16:0, 16:1ω7, 18:0 and 18:1ω9. The fatty acids in alkyldiacylglycerol were 16:0, 18:0, 22:5ω3 and 22:6ω3. Alkyldiacylglycerol was particularly rich in docosahexaenoic acid (22:6ω3) representing 30% of the total fatty acids. The alcohols of wax ester were all saturated with C_{20}-C_{29} carbon chains. The deacylated products derived from alkyldiacylglycerols were identified as hexadecyl, octadecyl and octadec-9′-enyl glycerol ethers.
Lipids 27, 75–78 (1992).

The sperm surface undergoes marked alterations during the various developmental stages of the male gametes, such as epididymal maturation, uterine capacitation, and acrosomal reaction and fertilization that take place in the fallopian tube (1,2). The cell surface of spermatozoa is believed to play a vital role at these stages, although specific surface molecules that regulate sperm motility and fertility have not yet been identified (1,2). Lipids, being major constituents of the cell surface, may play a role in regulating cellular recognition, transmembrane signalling, species specificity and tissue immunity (3–5). The lipids of boar (6) and ram (7) sperm plasma membrane (PM) have been found to change markedly as the cells undergo maturation during the epididymal transit. These studies suggest that cell surface lipids may have a role in regulating sperm maturation.

While analyzing goat sperm PM lipids, two unusual neutral lipids were detected. This study describes their identification as wax esters and 1-*O*-alkyl-2,3-diacylglycerols (Alkyl-DAG).

MATERIALS AND METHODS

Materials. Polyethyleneglycol (15–20 KDa), dextran (average mol. wt. 229000), hog pancreatic lipase, 1-*O*-hexadecylglycerol, 1-*O*-octadecylglycerol and 1-*O*-hexadecyl-2,3-dipalmitoyl-*sn*-glycerol were obtained from Sigma Chemical Company (St. Louis, MO). Palmityl oleate was prepared synthetically (8). Trisil-Z was a product of Pierce Chemical Company (Rockford, IL). The polymer system containing 5.5% dextran and 4.2% polyethyleneglycol in 0.1M K-phosphate, pH 6.5, was prepared as reported previously (9). Spermatozoa were extracted from goat epididymides within 2–4 hr after slaughter of the animals.

Isolation of goat sperm plasma membrane. Highly motile spermatozoa were extracted from goat cauda-epididymides in a modified Ringers phosphate buffered saline medium that contained 119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, 16.3 mM K-phosphate, pH 6.9, and penicillin (50 units/mL) (10). PM was isolated by the aqueous two-phase polymer method described earlier (9). Our previous study had demonstrated that the membranes isolated by this method are very pure, as judged by electron microscopy and analysis of marker enzymes (9). Electron microscopy showed the predominantly vesicular structure of sperm membranes (see Fig. 4 in ref. 9). The specific activities of alkaline phosphatase and 5′-nucleotidase as plasma membrane markers were approximately 14-fold higher in the isolated membrane than in sperm homogenate. The membrane had no detectable activity of acrosin and glucose-6-phosphatase, the markers of the acrosome and endoplasmic reticulum, respectively. The specific activity of cytochrome oxidase, an enzyme marker of mitochondria, was nearly seven-fold lower in the membranes than in the sperm homogenate. These data demonstrate that the isolated sperm plasma membranes were barely contaminated with acrosomes, endoplasmic reticulum, or mitochondria (see Table 4 in ref. 9).

Isolation of neutral lipids. Total lipids of the sperm PM were extracted following the method of Folch *et al.* (11). The neutral lipids were separated from other lipids by silicic acid column chromatography according to Rouser *et al.* (12).

Fractionation of neutral lipids by thin-layer chromatography. Neutral lipids were fractionated by preparative thin-layer chromatography (TLC) (13,14). Preliminary identifications were done by comparing R_f values with those of reference compounds applied to the same plate. For identification of wax esters (WE) and Alkyl-DAG, synthetic palmityl oleate and 1-*O*-hexadecyl-2,3-dipalmitoyl-*sn*-glycerol, respectively, were used. The alkyldiacylglycerol fraction was further purified by preparative TLC (15) using petroleum hydrocarbon/benzene (95:5, v/v) as developing solvent.

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Abbreviations: Alkyl-DAG, 1-*O*-alkyl-2,3-diacylglycerol; DAG, diacylglycerol; GLC, gas-liquid chromatography; PM, plasma membrane; TLC, thin-layer chromatography; TMS, tetramethylsilane; WE, wax ester.

Analysis of wax esters. Wax esters were hydrolyzed on TLC plates according to Misra *et al.* (8) using lipid-free (16) hog pancreatic lipase. After recovery of the alcohols and fatty acids, alcohols were analyzed as acetates and fatty acids as methyl esters by gas-liquid chromatography (GLC) as described previously (17,18).

Analysis of 1-O-alkyl-diacylglycerols. The Alkyl-DAG were refluxed with methanolic hydrogen chloride for 2 hr under anhydrous conditions (15), and the methyl esters and 1-*O*-alkylglycerols formed were separated by preparative TLC. Aliquots of 1-*O*-alkylglycerols were silylated using Trisil-Z and analyzed by GLC on a 3% OV-17 column (15). Authentic 1-*O*-alkylglycerol standards *viz.*, 1-*O*-hexadecyl-*sn*-glycerol and 1-*O*-octadecyl-*sn*-glycerol, were derivatized and used as GLC standards.

A portion of the 1-*O*-alkylglycerol fraction was acetylated (19) and subjected to argentation TLC on layers impregnated with 10% silver nitrate (20). Two bands were recovered, one corresponding to 1-*O*-hexadecyl-2,3-diacetyl-*sn*-glycerol (R_f 0.6) prepared by acetylation of authentic 1-*O*-hexadecyl-*sn*-glycerol, the other with a lower migration rate (R_f 0.45) containing one double bond. Portions of both bands, along with their catalytically reduced (21) products, were analyzed by GLC. A portion of the compound with R_f 0.45 was oxidized by permanganate/periodate reagent and the methyl esters of monocarboxylic acid were recovered from the TLC plate and analyzed by GLC (20).

Infrared (IR) and ^1H nuclear magnetic resonance (NMR) spectroscopy. ^1H NMR spectra of Alkyl-DAG were recorded on a JEOL FX-100 FT (Tokyo, Japan) instrument using $CDCl_3$ as solvent with tetramethylsilane (TMS) as internal standard. IR spectra of Alkyl-DAG were recorded in nujol on a Shimadzu (Tokyo, Japan) IR-408 spectrophotometer.

RESULTS

Two uncommon lipid bands were detected when the neutral lipids from sperm plasma membrane were analyzed by TLC. The R_f values of the bands were 0.85 and 0.68 when petroleum hydrocarbon/diethyl ether/glacial acetic acid (90:10:1, by vol) was used as the developing solvent. As described below, the PM-bound neutral lipids were identified as WE (R_f 0.85) and Alkyl-DAG (R_f 0.68). Sperm PM contained approximately 3 and 5 μg/mg protein of WE and Alkyl-DAG (Table 1), or 1.5% and 2.4% of total neutral lipids, respectively. The compounds were identified by comparison of their R_f values with those of the authentic standards. The structures were further confirmed by IR spectroscopy. IR showed characteristic strong ester bands at 1735 and 1180 cm^{-1} for both compounds and an *O*-alkyl ether band at 1110 cm^{-1} for Alkyl-DAG. The ^1H NMR (100 MHz) spectrum of alkyldiacylglycerol showed signals at 5.34 ppm (*m*, C*H* = C*H*), 5.18 (*m*, H-2), 4.24 (AB part of an ABX spectrum with J_{AB} = 12 Hz, J_{AX} = 6 Hz, J_{BX} = 4 Hz; H-3), 3.54 (*d*, *J* = 6 Hz, H-1), 3.4 (*t*, *J* = 6 Hz, H-1), 2.64 (*t*, *J* = 4 Hz, = CH-CH_2-CH=), 2.3 (*t*, *J* = 7 Hz, -CH_2COO), 2.04 (*m*, -CH_2-CH=CH-), 1.24 (broad *s*, CH_2) and 0.88 (*t*, *J* = 6 Hz, CH_3-CH$_2$). The ^1H NMR spectrum was in good agreement with that reported by Wood and Snyder (22), and more recently by Pakrashi *et al.* (23). Lipase hydrolysis (8) of wax esters yielded fatty acids and fatty alcohols which were identified and quantitated by GLC analysis and were found to be present in a 1:1 molar ratio. Hydrolysis of Alkyl-DAG yielded fatty acids and 1-*O*-alkylglycerols which were identified and quantitated by GLC analysis, and were found to be present in 2:1 molar ratio.

The trimethylsilyl derivatives of the 1-*O*-alkylglycerols gave three peaks in GLC analysis, two of which were identified as 1-*O*-hexadecyl- and 1-*O*-octadecylglycerols by comparison of their retention times with authentic compounds. The peak that appeared ahead of 1-*O*-octadecylglycerol was identified following argentation TLC. The acetylated 1-*O*-alkylglycerols were separated into two bands by argentation TLC. The band with R_f 0.6 yielded two peaks in GLC, corresponding to the acetyl derivatives of 1-*O*-hexadecyl- and 1-*O*-octadecylglycerol. The other band with R_f 0.45 showed IR absorption bands at 3030 and 1650 cm^{-1}, indicating the presence of *cis*-double bonds. Upon catalytic reduction, this band yielded 1-*O*-octadecyl-2,3-diacetate, having a retention time in GLC identical to that of authentic compound prepared by acetylation of 1-*O*-octadecylglycerol. Oxidation of the R_f 0.45 band with permanganate/periodate and analysis of the monocarboxylic fatty acid as methyl ester by GLC produced C-9 (9:0, nonanoic) acid. The data confirmed that the compound was 1-*O*-*cis*-octadec-9′-enylglycerol.

Table 2 shows the fatty acid composition of WE and Alkyl-DAG of sperm PM. The lipids contained fatty acids having chain lengths of C_{14} to C_{22}. The major fatty acids of the WE were 14:0, 16:0, 16:1ω7, 18:0 and 18:1ω9, whereas Alkyl-DAG were particularly rich in fatty acids having chain lengths of 16:0, 18:0, 22:5ω3 and 22:6ω3. The Alkyl-DAG possessed a markedly lower ratio of saturated/unsaturated fatty acids than the WE, primarily because of its unusually high content of docosahexaenoic acid (22:6ω3).

The composition of the alcohols derived from WE measured upon lipolysis is presented in Table 3. Carbon chain lengths were found to be from 20- to 29 carbons; major components were 24:0, 28:0 and 29:0 alcohols.

TABLE 1

Wax Ester and 1-*O*-Alkyldiacylglycerol Contents of Sperm Plasma Membrane Lipids[a]

Components	Lipid contents (μg/mg protein)
Total lipid	800 ± 2
Neutral lipid	216 ± 16 (27.6 ± 2.1)[b]
Wax ester	3.2 ± 0.04 (1.5 ± 0.02)[c]
1-*O*-Alkyl-2,3-diacylglycerol	5.2 ± 0.86 (2.4 ± 0.4)[c]

[a]Data are means of three experiments on three isolates. Each isolate was obtained by pooling the sperms from the cauda region of the epididymis from 5–10 animals.

[b]Values within parentheses show % (w/w) of total lipids.

[c]Values within parentheses show % (w/w) of neutral lipids.

DISCUSSION

Wax esters and ether lipids, including Alkyl-DAG, are known constituents of mammalian cells (24,25). Plasma membranes of mammalian cells contain ether-linked

TABLE 2

Fatty Acid Compositions of Wax Esters and 1-*O*-Alkyldiacylglycerols Isolated from Plasma Membrane of Goat Sperm[a]

Fatty acids[b]	Wax esters	1-*O*-Alkyldiacylglycerols
14:0	8.8 ± 2.0	6.4 ± 0.5
14:1	—	2.3 ± 0.8
16:0	24.1 ± 1.1	20.7 ± 0.7
16:1ω7	9.2 ± 0.8	0.6 ± 0.05
18:0	13.4 ± 1.1	9.8 ± 0.6
18:1ω9	15.2 ± 0.9	5.0 ± 0.08
18:2ω6	6.0 ± 1.0	1.8 ± 0.9
18:3ω3	5.3 ± 0.05	1.8 ± 0.08
20:0	0.1 ± 0.002	—
20:4ω6	4.4 ± 0.9	4.2 ± 0.08
20:5ω3	2.0 ± 0.8	1.3 ± 0.002
21:5ω3	—	1.8 ± 0.03
22:0	3.2 ± 0.03	—
22:2	—	0.2 ± 0.011
22:5ω6	2.1 ± 0.05	4.1 ± 0.8
22:5ω3	2.0 ± 0.003	10.0 ± 1.2
22:6ω3	4.2 ± 1.2	30.0 ± 2.8
Saturated/Unsaturated ratio	0.98	0.58

[a]Expressed as % (w/w) of total fatty acids.

[b]Data are means of three experiments on three isolates (see Table 1).

TABLE 3

Alcohol Composition of Wax Esters Isolated from Plasma Membrane of Goat Sperm[a]

Alcohols	Percent[b]
20:0	2.6 ± 0.05
22:0	2.6 ± 0.05
23:0	1.6 ± 0.02
24:0	41.7 ± 1.8
25:0	0.3 ± 0.004
26:0	4.9 ± 0.3
27:0	1.0 ± 0.001
28:0	24.6 ± 2.6
29:0	20.7 ± 1.5

[a]Data are means of three experiments on three isolates (see Table 1).

[b]Expressed as % (w/w) of total alcohol.

phospholipids and glycolipids (6,7). The present study reports the presence of both WE and Alkyl-DAG in PM of a mammalian cell type, namely the spermatozoon. The sperm PM preparations we used were highly pure, as evidenced by electron microscopy and marker enzyme analysis (9). It thus appears that contaminating trace amount of cellular organelles, if any, would not make an appreciable contribution to the observed WE and ether lipid contents of isolated sperm PM.

Little is known about the biological significance of these neutral lipids. However, it is known that ether linkages resist hydrolytic action by lipases (26) and are likely to retard the rate of hydrolysis of acyl moieties next to the ether bond (27).

Phosphatidylinositol and phosphatidylcholine are known to be involved in transmembrane signalling in response to various external stimuli such as hormones or neurotransmitters (4,5). DAG, as a second messenger, activates protein kinase C which, in turn, propagates the signal by phosphorylation of specific proteins (4). Stimulation of MDCK cells by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate results in the production of 1-*O*-alkyl-2-acyl-glycerols by the phospholipase C mediated hydrolysis of phosphatidylcholine (28). 1-*O*-Hexadecyl-2-acetyl-*sn*-glycerol, a synthetic alkylacylglycerol, has been found to inhibit the growth of HL-60 promyelocytic leukemia cells and to stimulate their differentiation to macrophage-like cells (29). Recently Daniel *et al.* (30) have shown that the synthetic 1-*O*-alkyl-2-acylglycerols inhibit DAG-activated protein kinase C activity. Furthermore, it has been speculated that the biological activity of alkylacylglycerol may be expressed through its inhibitory action on protein Kinase C (30). Alkylacylglycerol could also be generated in PM by the action of lipases on Alkyl-DAG, and affect signal transduction.

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Incorporation of Cyclic Fatty Acid Monomers in Lipids of Rat Heart Cell Cultures

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Primary cultures of newborn rat cardiomyocytes were grown in medium supplemented with cyclic fatty acid monomers (CFAM) which had been isolated from heated linseed oil. The cells were harvested, and lipids were extracted and fractionated using silica cartridges and high-performance liquid chromatography. The CFAM structures isolated from cellular lipids were determined and compared to those that had been supplemented to the medium, using gas-liquid chromatography coupled with mass spectrometry (GC/MS). We found that CFAM were incorporated into phospholipids and neutral lipids of cardiomyocytes. Furthermore, CFAM with a cyclopentyl ring structure were more abundant in cardiomyocytes than were the cyclohexyl ring isomers. Our data suggest that CFAM of the 5-carbon and 6-carbon ring series are metabolized differently in newborn rat cardiomyocytes.
Lipids 27, 79–81 (1992).

Oils and fats undergo chemical changes during heating, such as oxidation, polymerization, hydrolysis, isomerization and cyclization (1–4). Among the compounds formed, cyclic fatty acid monomers (CFAM), especially those formed from linolenic acid (5), are suspected to cause adverse health effects (6). In recent experiments with rats, high perinatal mortality was observed in pups whose mothers were fed high quantities of this type of CFAM (7). Other physiological effects of oils heated under extreme conditions may also be attributed to CFAM (8,9). Bird *et al.* (10,11) studied the effects on heart cell cultures of distillable, non-urea adductable fractions obtained from thermally oxidized fats. However, the composition of this fraction was not given and may have included oxidized components as well. Use of purified fractions of CFAM seems to be desirable to study the physiopathological effects of heated oils. However, feeding experiments require large quantities of purified material which would be time-consuming and expensive to prepare. Hence, cell cultures can serve as useful models.

As part of our ongoing work on the toxicity of different types of CFAM, the present study examines the incorporation of purified CFAM derived from linseed oil into heart cells.

MATERIALS AND METHODS

Cyclic fatty acid monomers were prepared from linseed oil heated at 275°C under nitrogen for 12 hr, as previously described (12). The oil was saponified, and the free fatty acids were esterified and purified from polar compounds (mainly polymers) by column chromatography on silicic acid. Urea fractionation was used to obtain the CFAM (99% pure).

The culture medium was Ham's F10 basal medium supplemented with 10% fetal bovine serum (Seromed, München, Germany) and 10% human serum (CTS, Dijon, France). CFAM in ethanol (100 mg/5 mL) were added to the medium using lipid-free bovine serum albumin (fraction V, Sigma, St Louis, MO) at 37°C, at a molar ratio CFAM/albumin of 6:1 (13). The medium containing the CFAM was then sterilized by filtration (Millex GS 0.22 μ, Millipore, Milford, MA).

Primary cultures of rat ventricular cells were prepared as previously described (14). The hearts from 2- to 4-day-old rats were aseptically removed, minced and washed 3 times in a cold Saline G solution (NaCl 8 g/L, KCl 0.4 g/L, KH_2PO_4 0.15 g/L, Na_2HPO_4 0.19 g/L and glucose 1.1 g/L; Prolabo, Paris, France), and one more time in the same solution for 10 min at 30°C in a shaking water bath. The fragments were then submitted to a 7-step (10 min each) trypsinization process (Trypsin, Difco, Detroit, MI; 0.1% in Saline G) at 30°C. The supernatants of the last 6 proteolytic treatments were pooled and diluted v/v in culture medium. The muscle to non-muscle cell ratio was increased by a two-step (30 and 150 min) selective adhesion procedure. The final cell suspension was diluted to 4×10^5 cells/mL in culture medium and seeded in 60-mm plastic Petri dishes (5 mL/dish). Cultures were maintained at 37°C in a humidified atmosphere (5% CO_2, 19% O_2 and 76% N_2). The culture medium was renewed 24 hr after seeding and thereafter every 48 hr.

Cells were separated into two groups, and incubated either in the same medium or in the CFAM containing medium (21.5 mg/L) for two days. Cells were then harvested by scraping with a rubber policeman and pelleted by centrifugation.

Total lipids were extracted from the medium according to Folch *et al.* (15) with two modifications. First, 10 mL of medium were extracted with 75 mL of solvent mixture and 5 mL of a 0.73% solution of sodium chloride (which takes into account the sodium chloride content of the medium). Secondly, 1 mL of formic acid was added, as suggested by Savary and Constantin (16), to break calcium soaps. Total cell lipids were similarly extracted except that no formic acid was used.

Phospholipids were separated from non-phosphorus lipids using a silica cartridge (Sep-Pak, Waters, Milford, MA) as described by Juaneda and Rocquelin (17). Phospholipid classes were obtained using high-performance liquid chromatography (HPLC) and ultraviolet (UV) detection (18).

Fatty acids were transesterified using boron trifluoride/methanol (19). Fatty acid methyl esters (FAME) were hydrogenated in chloroform/methanol solution (2:1, v/v) using platinum oxide (Merck, Darmstadt, Germany) as catalyst under 3 bars of hydrogen pressure. CFAM were then isolated *via* urea inclusion (20) and analyzed by gas-

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Abbreviations: CFAM, cyclic fatty acid monomers; FAME, fatty acid methyl esters; GC/MS, gas-liquid chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; UV, ultraviolet.

TABLE 1

Cyclic Fatty Acid Monomers (CFAM) in Culture Medium and in Lipids of Rat Cardiomyocytes in Culture

	Total CFAM (% of total methyl esters)	Relative proportions of the major CFAM types[a] (% of total CFAM)					
		1	2	3	4	5	6
CFAM fraction		16.2	22.1	6.5	22.3	9.4	23.4
Culture medium	11.5	18.0	18.0	6.1	21.0	8.1	23.4
Neutral lipids of cardiomyocytes	4.6	23.4	42.5	7.8	11.0	8.0	7.3
Phospholipids of cardiomyocytes	2.0	31.6	37.9	6.3	9.5	9.3	5.3
Phosphatidylcholine	3.5	32.5	37.6	6.8	11.0	7.3	4.8

[a]Bold numbers refer to the CFAM structures shown in Figure 1. The values are the mean of two separate cultures (more than 100 Petri dishes each).

liquid chromatography/mass spectrometry (GC/MS). A Hewlett-Packard 5970 Mass Selective Detector coupled with a Hewlett-Packard 5890 gas chromatograph was used with a fused silica column (J and W Scientific, Rancho Cordo, CA) coated with DB Wax (30 m × 0.25 mm i.d., film thickness 0.5 μm). The temperature was programmed from 50 to 200°C at 20°C/min, held at 200°C for 25 min, then programmed from 200°C to 220°C at 20°C/min and held at 220°C until completion of analysis. Helium was the carrier gas, and splitless injection was used.

RESULTS AND DISCUSSION

The CFAM fraction we used in this study was obtained from heated linseed oil and contained approximately half cyclopentyl isomers and half cyclohexyl compounds (21). CFAM distribution in the medium was uniform, and the relative proportions of the CFAM structures in the medium (Table 1 and Fig. 1) resembled those in the original CFAM fraction. Cyclic fatty acid monomers were not detected in the lipids of cardiomyocyte control cultures or in the culture medium.

The cardiomyocytes grown in CFAM-containing medium exhibited high levels of CFAM in their cellular lipids (Table 1). Incorporation of CFAM into phospholipids was about half that incorporated into neutral lipids. The data also show that incorporation of CFAM is rather selective. The cyclohexyl isomers 4 and 6 were incorporated into neutral lipids and phospholipids to a lesser extent (15–18% of the total CFAM in cell lipids *versus* 44% in culture medium) than were other CFAM, similar to what has previously been observed in female rats and their offsprings fed the same CFAM (7). Differences in the incorporation of cyclopentyl *versus* cyclohexyl ring CFAM had been difficult to observe in earlier studies due to the poor separation of individual CFAM (22) by gas-liquid chromatography (GC) on packed columns.

The results presented in Table 1 illustrate the effect of ring size of the CFAM. However, other factors, such as *cis versus trans* configuration, or propyl *versus* butyl substitution on the ring can also contribute to the observed differences.

Among the methods used in the present study, the urea inclusion step is critical. When the straight-chain fatty acids are not removed by urea inclusion, a very large peak of methyl stearate can mask some of the CFAM (23).

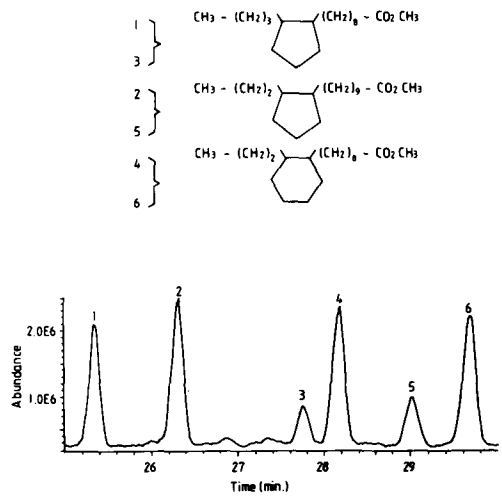

FIG. 1. Total ion chromatogram (GC/MS) of the hydrogenated CFAM from the cell culture medium. The structures are: 1 and 3, methyl 9-(2′-butylcyclopentyl)nonanoate; 2 and 5, methyl 10-(2′-propylcyclopentyl)decanoate; 4 and 6, methyl 9-(2′propylcyclohexyl)-nonanoate. Compounds 1, 2 and 4 have *trans* configuration, and compounds 3, 5 and 6 are the corresponding *cis* isomers.

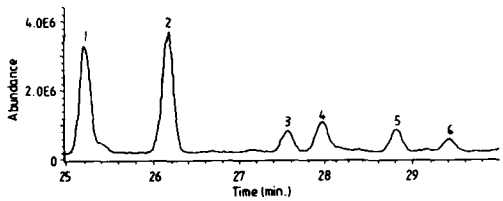

FIG. 2. Total ion chromatogram (GC/MS) of the hydrogenated CFAM derived from the phosphatidylcholine fraction of cultured rat cardiomyocytes. Peaks are identified as in Figure 1.

Sufficient enrichment of CFAM can be attained during urea inclusion when the proportions of urea and fatty acid methyl esters are at the optimum 3:1 ratio (7,12). When we attempted to quantify CFAM in individual phospholipid classes, accurate results were only obtained for the major phospholipid, namely phosphatidylcholine (Table

1, Fig. 2). For the lesser phospholipid classes, the large urea-to-fatty acid methyl ester ratio can cause CFAM losses due to trapping in the urea crystals. Special adaptation of the method will be required to accurately quantify low levels of CFAM in biological samples.

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Gamma Linolenic Acid (GLA) Content of Encapsulated Evening Primrose Oil Products

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The fatty acid composition of 16 brands of evening primrose oil (EPO) capsules was determined by capillary gas chromatography. Fourteen of these EPO brands contained γ-linolenic acid (GLA) levels between 7% and 10% (mean, 8.7; range, 1.9–10.5%) and there was generally good agreement between the level of GLA claimed by the manufacturer and the level determined by analysis. Low levels of the monoenes 22:1 and 24:1 found in some brands may indicate contamination of EPO with borage oil.
Lipids 27, 82–84 (1992).

There is evidence that, in humans, either the conversion of linoleic acid (LA, 18:2n-6) to γ-linolenic acid (GLA, 18:3n-6) is blocked or the Δ6 desaturase responsible for the conversion is fully saturated. In either event, levels of GLA and other metabolites, including dihomo-γ-linolenic acid (DGLA, 20:3n-6) and arachidonic acid (AA, 20:4n-6), are unaffected by dietary supplementation with LA rich oils, but are elevated by consumption of evening primrose oil (EPO) which contains on average 9% GLA (1).

A number of clinical conditions have been treated orally with EPO, including pre-menstrual tension (2), rheumatoid arthritis (3) and breast disorders (4). Atopic eczema in children and adults is the condition showing the best result from oral EPO treatment (5). Based on a number of such studies (6), a product license has been granted to at least one pharmaceutical company under the label "Epogam" for the treatment of eczema.

In many countries, including Australia, numerous EPO brands are available in pharmacies and health food stores. We report here a systematic study on the fatty acids of a range of EPO products which we measured using sensitive capillary column gas chromatography.

MATERIALS AND METHODS

EPO capsules were obtained from pharmacies and health food stores in Sydney, Melbourne and Adelaide and, where possible, more than one batch number of each product was purchased. All capsules tested were in the soft gelatin form. The retail cost varied and ranged from A$ 0.19 to A$ 0.52 per capsule.

All EPO samples were tested against a standard that had been extracted from evening primrose seed in our laboratory. Seeds (5 g) were homogenized in 30 mL of petroleum hydrocarbon and the homogenate centrifuged. The supernatant was decanted and the sediment extracted twice with 30-mL washes of petroleum hydrocarbon. The combined supernatants were filtered and the solvent evaporated. Thin-layer chromatography (TLC) revealed that only triglycerides were present.

Fatty acid analysis. TLC showed that all commercial products were predominantly in the triglyceride form. EPO was removed from capsules and 1–2 μL added to glass vials without further purification. Five mL of a solution of 1% H_2SO_4 in methanol was added to each vial. Vials were sealed and heated at 70°C for 3 hr. After cooling, the resulting methyl esters were extracted with 2 mL n-heptane. The extract was transferred into 2-mL auto sampler vials containing anhydrous sodium sulfate. Fatty acid methyl esters were separated on a wide bore (0.56 mm) 50-m capillary column coated with SP 2340 (Supelco Inc., Bellefonte, PA) using a Hewlett Packard 5880 gas chromatograph (Hewlett Packard, Palo Alto, CA). Chromatographic conditions were those described in detail elsewhere (7). Helium was used as the carrier gas at a flow of 30 cm/sec. The oven temperature was increased from 120°C to 200°C at 5°C per min; injection temperature was held at 250°C; and flame ionization detector temperature was 300°C. Samples (1 μL) were injected using the split mode at a 30:1 ratio.

Variation. All products were sampled in triplicate. Variation in major peaks was less than 5%, while the variation in minor peaks (<1% of total fatty acids) was less than 15%. Analyses not meeting these ranges were rejected.

RESULTS AND DISCUSSION

A total of 16 brands of EPO were analyzed (Table 1). Our aim was to examine at least three batches of each brand, but these were not always available. Of the EPO brands examined most contained levels of GLA between 7–10%; exceptions were one batch of Bioglan (6.3%) and Moonkist (1.9%). There was generally good agreement between the level of GLA claimed by the manufacturer and that determined by analysis. The levels of the major fatty acid components of all the products were consistent with the range of values seen for genuine EPO in our laboratory. The very low level of GLA (1.9%) in Moonkist, a locally grown product, is thought to be due to climatic conditions. The high levels (>20%) of saturates measured in Nature's Own Plus are due to oils present in an added vitamin/mineral mix.

Examination of the minor components of the products showed interesting differences between brands. Several brands contained detectable amounts of C_{22} and C_{24} monounsaturated fatty acids (MUFA), which were not found in our laboratory standard EPO. While it is possible that these low levels of C_{22} and C_{24} MUFA are due to genetic differences among the various strains of evening primrose, we consider this unlikely. Analysis of oil extracted in our laboratory from seeds of evening primrose strains from Europe, China, Australia, New Zealand and North America has failed to reveal the presence of these MUFA.

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Abbreviations: EPO, evening primrose oil; GLA, γ-linolenic acid (18:3n-6); LA, linoleic acid (18:2n-6); MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; TLC, thin-layer chromatography.

TABLE 1

Fatty Acid Composition[a] of Different Brands of Evening Primrose Oil Capsules

Brand[b]	Batch no.	Label GLA[c]	GLA	LA	Total SFA[d]	Total MUFA[e]	20:1n-9	22:1n-11	24:1n-9
Naudicelle	30924	10	9.8	72.2	8.5	9.3	0.13	—	—
	30130	10	9.6	71.2	8.9	10.2	0.21	—	—
	30927	10	9.2	71.4	9.4	9.8	0.18	—	—
Efamol	30234	8	9.5	71.9	8.4	10.1	0.12	—	—
	23038	8	8.2	71.1	8.1	12.4	0.13	—	—
	23634	8	8.2	73.9	8.8	8.9	0.16	—	—
Eviprim	24253	8	9.0	69.4	8.5	11.8	0.31	0.84	—
	29317	8	8.0	73.1	8.9	9.9	0.13	—	—
Blackmores	22994	8	7.6	71.2	8.1	13.1	0.14	—	—
	23600	8	8.6	74.1	8.3	8.9	0.15	—	—
	23749	8	8.0	74.3	8.8	8.9	0.16	—	—
Natural Nutrition	A0233	10	10.1	65.5	9.2	15.2	0.84	0.47	0.26
	A0139	10	10.1	65.5	9.2	15.2	0.83	0.47	0.26
	30305	10	9.1	65.5	9.3	16.1	0.80	0.46	0.24
Healtheries	90835	10	10.4	65.3	9.0	15.5	0.84	0.45	0.25
	00422	10	10.0	65.9	8.8	15.3	0.74	0.42	0.22
	2900	10	9.8	63.5	9.7	16.9	1.00	0.60	0.33
	1039	10	8.8	69.9	9.6	11.6	0.64	0.30	0.18
Nature's Own	M9830	10	10.0	63.2	9.2	16.2	0.88	0.52	0.29
	E0974	10	10.0	65.5	8.9	15.5	0.80	0.45	0.24
	M0093	10	9.0	65.3	9.4	16.4	0.81	0.47	0.26
Bioglan	5987B	8	9.3	74.3	8.4	7.9	0.14	—	—
	3261-2	8	8.2	74.7	8.3	8.7	0.15	—	—
	3064	8	6.3	67.7	10.4	15.3	0.21	0.09	—
Nature's Own Plus	32315	7.9	7.6	59.7	24.2	8.1	0.12	—	—
	32315	7.9	7.7	60.1	23.8	8.0	0.12	—	—
	3399A	7.9	7.4	64.5	26.2	9.9	0.16	—	—
	34941	7.9	7.1	63.0	26.3	11.1	0.16	—	—
Good Health	07	9–10	10.5	64.0	9.2	16.3	0.95	0.53	0.31
	08	10	10.5	64.0	9.2	16.2	0.91	0.53	0.30
Macro	33915	10	9.6	72.6	8.5	9.1	0.14	—	
	34562	10	9.5	73.3	9.2	7.6	0.15	—	—
Soul Pattinson	56727	8	7.6	74.1	8.9	9.3	0.16	—	—
Natural Care	16969	10	9.1	64.9	9.6	16.4	0.89	0.49	0.28
Amcal	36439	8	8.2	74.6	8.4	8.7	0.14	—	—
MicroGenics	8481	10	9.7	70.0	9.4	10.8	0.58	0.28	0.17
Moonkist EPO	190	5.2	1.9	75.8	13.7	8.6	0.15	—	—

[a] Values are means given as % weight of total fatty acids.

[b] The following products were sampled: Naudicelle (Key Pharmaceuticals Pty. Ltd. Rhodes, N.S.W. Australia); Efamol (Efamol, London, England); Eviprim (Key Pharmaceuticals Pty. Ltd.); Blackmores Botanicals (Blackmores Ltd. Balgowlah, N.S.W. Australia); Natural Nutrition, Nature's Own and Nature's Own Plus (Natural Health Products Pty. Ltd., Kippa-Ring, Q.L.D. Australia); Healtheries (Healtheries of New Zealand, Auckland, New Zealand); Bioglan [Bioglan Laboratories (Aust.) Pty. Ltd., Marrickville, N.S.W. Australia]; Good Health (Good Health Products, Auckland, New Zealand); Macro (Lederle Laboratories Division, Cyanamid Australia Pty. Ltd., Baulkham Hills, N.S.W. Australia); Soul Pattinson [Soul Pattinson (Laboratories) Pty. Ltd., Kingsgrove, N.S.W. Australia]; Natural Care Australia, Brunswick Heads, N.S.W. Australia); Amcal (Allied Master Chemists of Australia Limited, Springvale, V.I.C. Australia); Micro Genics (Twin Labs Pty. Ltd., Windsor, V.I.C. Australia); Moonkist (Gum Park Pty. Ltd., Cannawigara S.A. Australia).

[c] % As listed by manufacturer.

[d] Includes 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0.

[e] Includes 14:1, 15:1, 16:1, 17:1, 18:1, 20:1, 22:1 and 24:1.

TABLE 2

Fatty Acid Composition[a] of EPO, Borage and Safflower Oils

	GLA 18:3n-6	LA 18:2n-6	Total SFA[b]	Total MUFA[c]	20:1n-9	22:1n-11	24:1n-9
EPO	7.0	69.7	8.2	15.0	0.24	—	—
Borage	26.1	38.1	12.6	22.8	4.31	2.70	1.56
EPO/Borage (84:16)	9.8	64.9	8.8	16.4	0.79	0.46	0.25
Borage/Safflower (60:40)	9.8	60.9	10.8	18.3	1.94	1.27	0.90

[a] Values are means given as % weight of total fatty acids. [b] Includes 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0. [c] Includes 14:1, 15:1, 16:1, 17:1, 18:1, 20:1, 22:1, and 24:1.

We investigated the possibility that some of the capsules contained blends of EPO and either borage or safflower oil (Table 2). Starting with an EPO sample with 7% GLA, blends of oils were prepared which were designed to achieve a GLA level of 10%. The fatty acid profile of the EPO/Borage oil blend (84:16) more closely matched the profile seen in some of the capsules than the Borage/Safflower blend (60:40).

GLA is the claimed active ingredient of EPO and, thus, consumers and researchers alike are interested in the GLA content of EPO products. Although the levels of GLA in the products tested in this study were in the range expected for EPO, minor constituents in some products suggested that some may contain contaminants of other oils.

Unlike marine oils (8), EPO is relatively easy to analyze (particularly by capillary gas chromatography) as it contains only two major polyunsaturated fatty acids (LA and GLA), and sometimes low levels of α-linolenic acid (18:3n-3) EPO is devoid of C_{22} and C_{24} MUFA that are present in some other GLA-containing oils, such as borage (9). Despite this, several of the products that we tested were found to contain amounts of 20:1, 22:1 and 24:1 consistent with a blend of borage and EPO (Table 2). It is interesting that most of the capsules thought to contain EPO/borage blends also contained GLA levels close to 10% of the total fatty acids. Whether this represents an attempt by manufacturers to supply oils with a standard GLA content or is the result of accidental contamination during pressing and/or extraction is unknown.

There are no reports of the comparative physiological or clinical effects of the various GLA containing oils in humans. Until this is done, the significance of capsules of EPO containing low levels of borage or other oils remains largely one of labelling.

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Dietary Arachidonic and Linoleic Acids: Comparative Effects on Tissue Lipids

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The effects of preformed dietary arachidonic acid (AA, 20:4n-6) on murine phospholipid fatty acid composition in tissues capable (liver) and incapable (peritoneal exudate cells, PEC) of desaturating and elongating linoleic acid (LA, 18:2n-6) to AA were investigated. The results were compared with those obtained on matched animals on LA diets by either substituting or supplementing dietary LA with AA. Modest amounts of AA ethyl ester (0.5 wt%) included in the diet significantly increased tissue phospholipid AA levels by 39% and 57% in the liver and in PEC, respectively. The changes were further enhanced when dietary LA and AA intakes were equivalent, *i.e.*, 57% and 68% in liver and PEC, respectively. This enrichment was observed in all phospholipid classes analyzed, with the greatest impact on phosphatidylcholine. In addition, the doubling of dietary LA had little effect on tissue phospholipid AA levels. The data suggest that while the level of n-6 PUFA may have an important effect on tissue fatty acid composition, the type of n-6 PUFA in the diet could be of greater significance.
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Arachidonic acid (AA, 20:4n-6) is one of the most abundant polyunsaturated fatty acids (PUFA) associated with mammalian phospholipids. While dietary n-6 PUFA are essential and beneficial, elevated intakes may contribute to the pathologies associated with a number of chronic diseases (1,2). When AA is liberated from membrane phospholipids, it can be enzymatically oxidized to a variety of eicosanoids, many of which are proinflammatory and proatherogenic (3–6).

The conversion of linoleic acid (LA, 18:2n-6) to AA is regulated by a number of mechanisms (7–11). Tissue AA levels may be influenced by the amount of LA consumed in the diet (12). LA is desaturated (*via* the Δ5 desaturase and Δ6 desaturase) and elongated to AA, primarily in the liver. As dietary LA intake increases, tissue AA levels also increase, but this process appears to be saturable (12–15). AA is an inhibitor of both the Δ5 and Δ6 desaturases (10,11); thus, it is conceivable that when AA is included in the diet, tissue AA levels could remain constant at the expense of desaturation and elongation of LA. However, the effects that preformed dietary AA has on the fatty acid composition of tissues capable (*e.g.* liver) and incapable (*e.g.*, peritoneal exudate cells, PEC) of converting LA to AA are not clear (16). This is an important consideration as the estimated daily intake of dietary AA in the United States is approximately 170 mg and, based on the AA content of commonly consumed foods, this intake could easily be tripled (17,18).

Few studies have evaluated the dietary effects of AA on tissue fatty acid composition. Mohrhauer and Holman (12) examined the effects of dietary AA on total hepatic fatty acid composition in the rat. However, AA ethyl ester administered orally *via* microsyringe was the only lipid provided in the dietary regimen (12). Studies on human subjects either failed to control the fatty acid content of the diet (19), provided no tissue fatty acid composition data (20), or examined plasma lipid composition only (19,21,22). Therefore, in the present study we have evaluated the effects of dietary AA on hepatic and extrahepatic (*viz.*, PEC) tissue fatty acid composition and compared the results with those obtained on matched animals on LA diets by either substituting or supplementing dietary LA with AA.

METHODS

Animals. Twenty CD-1 male mice (Charles River, Wilmington, MA) (18–20 g) were, upon arrival, randomly divided into four dietary groups (diets 1–4), five animals per group. All mice were maintained on Prolab Chow diet (Agway, RMF 1000, Syracuse, NY) for one week. Because the chow diet contained a small amount of 18:3n-3, an inhibitor of Δ6 desaturase, the mice were placed on a fat-free diet (ICN Biochemicals, Cleveland, OH) for one week, at which time they were placed on their experimental diets for two weeks.

Diets. All diets contained 10.5 wt% fat. The LA content of the diets, as supplied primarily by safflower oil (75% LA) (Hollywood Foods, Los Angeles, CA), was maintained at 2.0 wt% (Tables 1 and 2). The filler oils (6.5 wt%) consisted of high oleic acid sunflower oil (77% 18:1n-9) (SVO, Eastlake, OH) and tripalmitin (99% 16:0) (Sigma, St. Louis, MO) (Table 2). Each of the four diets contained an additional 1.5 wt% of fatty acid ethyl esters (NuChek Prep, Elysian, MN). Diets 1, 2 and 4 contained the ethyl esters of oleic acid (18:1n-9), linoleic acid (LA) and arachidonic acid (AA) respectively, while diet 3 contained 1.0 wt% 18:1n-9 plus 0.5 wt% AA. Diets were prepared in bulk, prepackaged in separate Whirl-Pak bags (NASCO, Atkinson, WI) and stored under nitrogen at −20°C. Water and food were provided *ad libitum* for two weeks. Fresh diets were provided daily and uneaten food was discarded to minimize oxidation prior to consumption. The fatty acid analysis of the diets is presented in Table 2.

Fatty acid analysis. Following two weeks on the experimental diets, the animals were sacrificed by diethyl ether inhalation and the peritoneal cavity was immediately washed (twice) with saline (0.9%) containing 1 mM ethylenediaminetetraacetic acid (EDTA). The peritoneal exudate cell population consisted primarily of resident macrophages (95%) and some mast cells (<5%). The cells were differentiated according to standard morphological

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Abbreviations: AA, arachidonic acid; EDTA, ethylenediaminetetraacetic acid; LA, linoleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEC, peritoneal exudate cells; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography.

TABLE 1

Experimental Design

Diet	Dietary groups 1 Oleic acid	2 Linoleic acid	3 Oleic acid + arachidonic acid	4 Arachidonic acid
n	5	5	5	5
Safflower oil[a]	25[b]	25	25	25
High oleic sunflower oil[c]	50	50	50	50
Tripalmitin	15	15	15	15
Oleic acid ethyl ester[d]	15	—	10	—
Linoleic acid ethyl ester[d]	—	15	—	—
Arachidonic acid ethyl ester[d]	—	—	5	15

[a]Contains 75% 18:2n-6.

[b]g/kg diet.

[c]Contains 4% 18:2n-6.

[d]99% pure.

TABLE 2

The Fatty Acid Composition of the Diets

Fatty acids	Dietary groups 1	2	3	4
14:0	0.2[a]	0.2	0.2	0.2
16:0	16.5	16.9	16.5	16.1
18:0	4.5	4.5	4.6	4.6
18:1	58.1	38.6	51.2	40.0
18:2n-6	20.5	39.7	20.7	20.9
20:4n-6	—	—	6.7	18.4

[a]g/kg diet.

criteria using cytospin preparations stained with May-Grünwald-Giemsa stain. The peritoneal washes were pooled and centrifuged (700 × *g* for 4 min at 25°C) to pellet the PEC. The livers were perfused with cold (4°C) saline, removed, and homogenized in saline. The liver homogenates and pelleted PEC were resuspended in equal volumes of saline and chloroform/methanol (1:2, v/v) and the lipids were extracted sequentially with chloroform/saline (1:1, v/v) and chloroform (twice). The pooled extracts were evaporated to dryness under nitrogen and redissolved in chloroform. The PEC and liver phospholipids were separated from the neutral lipids by thin-layer chromatography (TLC) using a chloroform/methanol (8:1, v/v) solvent system. Fractions were made visible with 0.1% 8-hydroxy-1,3,6-pyrene-trisulfonic acid trisodium salt (Eastman Kodak, Rochester, NY) in methanol. The phospholipids were recovered from TLC plates by scraping the appropriate bands and resuspending in toluene. They were then saponified with 0.5 N KOH in methanol for 8 min at 86°C. Following acidification with 0.7 N HCl, the free fatty acids were extracted with equal volumes of hexane (twice), evaporated under nitrogen, and methylated with diazomethane. The fatty acid methyl esters were resuspended in hexane and analyzed by gas chromatography on a DB23 capillary column (0.25 mm × 30 m) (J&W Chromatography, Folsom, OH) with hydrogen as the carrier gas. The internal standard, pentadecanoic acid methyl ester, was added to each sample prior to the saponification step.

Fatty acid analysis of hepatic phospholipid classes. Following TLC separation of the hepatic phospholipids, the phospholipid classes were separated by a second TLC step using chloroform/methanol/acetic acid/water (25:20:3:0.3, by vol). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol plus phosphatidylserine (PI + PS) were identified by comparison with standards. The appropriate bands were scraped from the TLC plates and the fractions were resuspended in toluene. The fatty acid composition of the isolated phospholipid classes was determined by the methods described above.

Statistical analysis. The analytical data were statistically evaluated by Duncan's protected least significant difference test using the MSUSTAT statistical package (Montana State University, Bozeman, MT). Significance was determined at $P<0.05$.

RESULTS

Food intake and weight gain were not significantly different between any of the dietary groups.

Fatty acid composition of liver total phospholipids. When dietary LA was doubled from 2 wt% (diet 1) to 4 wt% (diet 2) by adding LA ethyl ester, the LA content in liver phospholipids increased from 18.3 mol% to 21 mol%, respectively, with very little change in AA content or long chain n-6 PUFA content (C_{22} PUFA) (Table 3). However, when AA was included in the diet at 0.67 wt% (diet 3), hepatic phospholipids were significantly enriched in AA at the expense of LA (Table 3). The AA content of the tissue phospholipids from animals on diet 3 increased by 32% when compared to that of animals on diet 2, despite 30% less total n-6 PUFA in the diet. When AA was equivalent to the amount of LA in the diet (diet 4), tissue AA levels were enriched above those levels observed in the other three dietary groups; *i.e.*, there was a 57%, 48% and 13% increase *vs* diets 1, 2 and 3, respectively.

Fatty acid composition of liver phospholipid classes. When LA was increased in the diet (diet 1 *vs* diet 2), LA and AA levels in all phospholipid classes (PC, PE and PI + PS) changed little (Table 4), particularly in PE and PI

TABLE 3

The Fatty Acid Composition of Phospholipids from Livers and Resident Peritoneal Cells[a]

	Liver				Peritoneal cells			
	Dietary groups				Dietary groups			
Fatty acid	1	2	3	4	1	2	3	4
16:0	19.6 ± 0.6^{b}	18.5 ± 1.2^{b}	21.3 ± 0.3^{c}	22.5 ± 0.5^{c}	25.5 ± 0.7^{b}	25.2 ± 0.3^{b}	25.7 ± 0.3^{bc}	26.4 ± 0.6^{c}
18:0	11.9 ± 0.6^{b}	12.9 ± 0.5^{b}	14.3 ± 0.5^{c}	13.6 ± 0.5^{bc}	17.2 ± 0.3^{b}	17.2 ± 0.2^{b}	16.9 ± 0.4^{b}	18.1 ± 0.6^{b}
18:1	17.8 ± 1.0^{b}	14.2 ± 0.5^{c}	12.7 ± 0.4^{c}	9.7 ± 0.5^{d}	24.6 ± 0.5^{b}	20.9 ± 0.2^{c}	20.8 ± 0.6^{c}	18.9 ± 1.3^{d}
18:2n-6	18.3 ± 0.7^{b}	21.0 ± 0.6^{c}	12.3 ± 0.4^{d}	10.1 ± 0.2^{e}	9.4 ± 0.3^{b}	10.4 ± 0.4^{c}	6.2 ± 0.3^{d}	4.5 ± 0.2^{e}
20:4n-6	21.3 ± 0.3^{b}	22.5 ± 0.6^{b}	29.6 ± 0.5^{c}	33.4 ± 0.2^{d}	11.3 ± 0.6^{b}	12.7 ± 0.1^{b}	17.7 ± 0.6^{c}	19.0 ± 0.7^{c}
22:4n-6	—[f]	—	—	1.6 ± 0.1	2.8 ± 0.3^{b}	4.1 ± 0.2^{bc}	5.5 ± 0.5^{c}	6.3 ± 0.9^{c}
22:5n-6	2.8 ± 0.3^{b}	3.6 ± 0.3^{bc}	3.7 ± 0.4^{bc}	4.2 ± 0.2^{c}	1.3 ± 0.1^{b}	1.8 ± 0.1^{c}	1.6 ± 0.1^{bc}	1.5 ± 0.1^{bc}
22:6n-3	2.8 ± 0.3^{bc}	2.3 ± 0.2^{b}	3.2 ± 0.2^{c}	3.1 ± 0.1^{c}	1.0 ± 0.1^{b}	—	1.1 ± 0.1^{b}	1.0 ± 0.1^{b}

[a]Mol% (Columns do not total 100% as those fatty acids contributing less than 1 mol% were not included.). Mean ± SEM of five experimental values. Means with the same superscript within the same row are not significantly different at $p<.05$, Duncan's protected least significant difference.

[f]Less than 1 mol%.

+ PS, with a slight but significant increase in PC. When AA was included in the diet, the relative content of AA almost doubled in PC of mice on diet 3 compared to diet 1 or diet 2, with a further increase of AA content in animals on diet 4. The LA levels in all phospholipid classes were approximately halved after the introduction of AA to the diet, with the greatest change also occurring in PC.

Fatty acid composition of peritoneal cell phospholipids. Dietary AA was very effective in altering the phospholipid fatty acid composition of the resident PEC (Table 3). The AA levels in PEC progressively increased with increasing dietary AA consumption, apparently at the expense of LA. Significantly, the doubling of dietary LA resulted in no significant change in peritoneal cell AA content.

DISCUSSION

Tissue AA can be derived from dietary LA *via de novo* synthesis, and this conversion appears to be highly regulated (7–11). This study showed that the doubling of dietary LA (diet 1 *vs* diet 2) resulted in a slight but insignificant increase in tissue AA levels in both the liver and PEC. Thus, as LA increased in the diet, its conversion to AA and eventual incorporation into tissue phospholipids was saturable. However, when AA was included in the diet, its effect on tissue phospholipid fatty acid composition was significant. Even when the dietary AA intake was one-third that of LA (diet 3 *vs* diet 2), tissue AA levels were significantly higher in the AA dietary group (diet 3), 32% and 39% in the liver and in PEC, respectively. These changes were even greater when dietary LA and AA intakes were equivalent (diet 1 *vs* diet 4), *i.e.*, 57% and 68% in the liver and in PEC, respectively. It is important to note that most of the effects of dietary n-6 PUFA on tissue 20:4n-6 levels came as a result of including modest amounts of 20:4n-6 in the diet (diet 3), with only a moderate increase when dietary 20:4n-6 was increased three-fold (diet 4). Therefore, while the level of dietary n-6 PUFA may be an important influence on tissue phospholipid fatty acid composition, the type of n-6 PUFA in the diet could be of greater significance (diet 2 *vs* diet 4).

Increasing dietary LA had little effect on AA content of hepatic phospholipid classes in any of the phospholipid fractions, but inclusion of AA in the diet resulted in significant enrichment of AA in all phospholipid classes. The data demonstrate that dietary AA is readily and preferentially incorporated into PC. This type of selectivity for AA has been observed elsewhere, under *in vitro* (13,23,24) and *in vivo* conditions (25).

Mice efficiently elongate and desaturate C_{18} PUFA to their C_{20} PUFA derivatives (26,27). Both mice and rats have relatively high Δ5 desaturase activity compared to a number of other species, including human (26). Low Δ5 desaturase activity would be one way of controlling tissue AA levels. Therefore, the impact of dietary AA on tissue AA content may be of great significance in those species which poorly elongate and desaturase LA to AA.

The enrichment of tissue phospholipids by dietary AA can be explained by a number of factors. Dietary AA does not have to be elongated and desaturated prior to tissue incorporation. Based on guinea-pig data, it has been estimated that as little as 0.01% of dietary LA may be converted to AA, with even a smaller percentage being incorporated into tissue phospholipids (28). Secondly, LA appears to be a relatively good substrate for β-oxidation in contrast to AA, which is a poor one. Studies evaluating the oxidation of a variety of ^{14}C-labelled PUFA revealed that only 14% of dietary AA is oxidized to [^{14}C]CO_2 (after 24 hr), in contrast to 48% for LA (25). Thirdly, C_{20} PUFA are preferentially incorporated into tissue phospholipids (13,23,24). The activities of liver acyl-CoA synthase and acyl CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase, key enzymes in phospholipid biosynthesis, exhibit relatively high specificity for AA when compared to other fatty acid, including LA (23).

In summary, the inclusion of AA in the diet, even in modest amounts, resulted in facile enrichment of tissue phospholipid AA content. This enrichment was observed in all phospholipid fractions (PC, PE, PS + PI), with the greatest impact on PC. Increasing dietary LA levels resulted in small increases in tissue AA content, but these changes were minimal compared with those observed with dietary AA. These results may be particularly relevant in species with limited desaturase activities, *e.g.*, human.

TABLE 4

The Fatty Acid Compositions of Phosphatidylcholine, Phosphatidylethanolamine and Phosphatidylinositol Plus Phosphatidylserine of Livers of Mice Fed Various n-6 PUFA Diets[a]

Fatty acid	Phosphatidylcholine				Phosphatidylethanolamine				Phosphatidylinositol plus phosphatidylserine			
	Dietary groups				Dietary groups				Dietary groups			
	1	2	3	4	1	2	3	4	1	2	3	4
16:0	27.5 ± 0.7^{b}	26.8 ± 0.3^{b}	26.6 ± 0.9^{b}	27.4 ± 1.4^{b}	18.3 ± 0.8^{b}	18.9 ± 0.4^{b}	18.2 ± 0.3^{b}	19.2 ± 0.9^{b}	8.3 ± 1.2^{b}	6.7 ± 0.4^{c}	5.9 ± 0.2^{c}	7.6 ± 1.6^{bc}
18:0	11.4 ± 0.4^{b}	12.1 ± 0.6^{bc}	12.8 ± 1.0^{bc}	13.3 ± 0.7^{c}	21.8 ± 1.0^{b}	25.3 ± 0.4^{c}	24.7 ± 0.6^{c}	24.6 ± 0.4^{c}	39.6 ± 2.0^{b}	45.4 ± 0.2^{c}	44.5 ± 0.5^{c}	42.0 ± 1.4^{d}
18:1	19.5 ± 1.2^{b}	15.9 ± 1.0^{c}	14.7 ± 0.8^{c}	9.6 ± 0.3^{d}	15.0 ± 1.9^{b}	11.2 ± 0.3^{c}	8.1 ± 0.6^{d}	6.1 ± 0.6^{e}	6.0 ± 0.5^{b}	4.2 ± 0.2^{c}	3.0 ± 0.2^{d}	2.3 ± 0.1^{e}
18:2n-6	19.0 ± 1.0^{b}	22.7 ± 0.8^{c}	11.1 ± 1.0^{d}	8.1 ± 0.4^{e}	7.6 ± 0.7^{b}	7.6 ± 0.5^{b}	4.3 ± 0.3^{c}	3.4 ± 0.3^{c}	4.0 ± 0.6^{b}	3.5 ± 0.3^{bc}	2.1 ± 0.2^{d}	1.9 ± 0.3^{d}
20:4n-6	13.5 ± 0.9^{b}	14.3 ± 0.5^{b}	27.6 ± 0.6^{c}	34.0 ± 1.4^{c}	25.9 ± 0.9^{b}	25.4 ± 0.4^{b}	30.7 ± 0.4^{c}	30.3 ± 0.2^{c}	31.5 ± 1.1^{b}	34.0 ± 0.5^{b}	41.1 ± 0.4^{c}	42.3 ± 0.9^{c}
22:5n-6	2.0 ± 0.2^{b}	2.3 ± 0.2^{bc}	3.0 ± 0.3^{cd}	3.3 ± 0.1^{d}	4.3 ± 0.4^{b}	5.5 ± 0.4^{bc}	6.6 ± 0.7^{cd}	7.8 ± 0.2^{d}	2.4 ± 0.4^{b}	1.2 ± 0.1^{c}	1.3 ± 0.1^{c}	1.5 ± 0.2^{c}
22:6n-3	1.7 ± 0.2^{b}	1.3 ± 0.1^{b}	2.4 ± 0.2^{c}	2.4 ± 0.1^{c}	4.2 ± 0.4^{b}	3.3 ± 0.3^{b}	5.4 ± 0.3^{c}	5.5 ± 0.2^{c}	1.9 ± 0.4^{b}	—^{f}	—	1.1 ± 0.1^{c}

[a]Mol% (Columns do not total 100% as those fatty acids contributing less than 1 mol% were not included.). Mean ± SEM of five experimental values. Means with the same superscript within the same row are not statistically different at $p<0.05$, Duncan's Protected Least Significant Difference.

[f]Less than 1 mol%.

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ARTICLE

Docosahexaenoic Acid in Developing Brain and Retina of Piglets Fed High or Low α-Linolenate Formula with and Without Fish Oil

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Docosahexaenoic acid (22:6n-3) can be synthesized in the liver and/or brain from α-linolenic acid (18:3n-3) and is required in large amounts in structural membranes of developing brain and retina. The adequacy and efficacy of formulas containing 18:3n-3 and/or fish oil in providing 22:6n-3 for deposition was investigated in piglets fed formula from birth to 15 days. The test formulas contained high (HL) or low (LL) 18:3n-3 (3.9 or 0.7% of the total formula fatty acids, respectively), or low 18:3n-3 plus fish oil (LL+FO) to provide C_{20} and C_{22} n-3 polyunsaturated fatty acids (0.8% of total fatty acids). Fatty acid analyses of synaptic plasma membrane and retina ethanolamine phospholipids (EPL), which are especially enriched in 22:6n-3, were compared to those of 15-day-old piglets fed sow milk (SM). Feeding LL resulted in lower 22:6n-3 in synaptic plasma membrane. Fatty acid levels in HL and LL+FO piglets were equivalent to SM, with the exception of lower 22:5n-3 in the synaptic plasma membrane of LL+FO and in the retina of HL and LL+FO-fed piglets. Levels of 22:4n-6 were also lower in the retina of the LL+FO group. The results suggest formula 18:3n-3 is at least 24% as effective as C_{20} and C_{22} n-3 fatty acids as a source of membrane 22:6n-3. This study shows dietary 18:3n-3, as the only n-3 fatty acid, can support deposition of comparable percentage of 22:6n-3 to natural milk. Fish oil also supported tissue levels of 22:6n-3 similar to natural milk; however, lower 22:4n-6 may indicate possible inhibitory effects on n-6 metabolism.

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Docosahexaenoic acid (22:6n-3) is required in large amounts in phospholipids (PL) of cellular and subcellular membranes of the developing brain and retina. Prolonged dietary deficiency of n-3 fatty acids may result in decreased brain and retina 22:6n-3 (1–8) and impairment of normal visual function in rodents (9) and monkeys (7). The visual problems seem to persist even when the low brain and retina 22:6n-3 levels are restored (7). Altered visual acuity also has been demonstrated in premature infants fed formulas providing less than 1% of total fatty acids as α-linolenic acid (18:3n-3) and no C_{20} or C_{22} polyunsaturated fatty acids (PUFA) (10,11). In humans, the period of neuronal differentiation and maturation coincides with rapid C_{20} and C_{22} PUFA accretion in structural PL of the central nervous system (CNS) (12). The functional alterations suggest that tissue levels of 22:6n-3 may be important for normal development of the CNS.

Human breast milk typically provides 22:6n-3 and other C_{20} and C_{22} n-3 and n-6 PUFA, as well as about 8–17% of total fatty acids as linoleic acid (18:2n-6) and 0.5–1% of total fatty acids as 18:3n-3 (13). Infant formulas provide 18:2n-6 and 18:3n-3 but, in contrast to milk, they contain little or no C_{20} and C_{22} PUFA. Low levels of 22:6n-3 in red blood cell (RBC) and plasma PL of infants fed formula have led to the speculation that the desaturase enzymes required for the synthesis of C_{20} and C_{22} derivatives of 18:2n-6 and 18:3n-3 may be immature at birth (14,15) or that C_{20} and C_{22} PUFA, if synthesized, are unavailable to growing neural tissues (16). This has led to suggestions that 22:6n-3 is essential in the diet of the newborn (17,18), and to clinical trials with fish oil-supplemented formulas (10,11,19). Although effective in supplying 22:6n-3 for developing tissues (3,7,20–23), the high amount of eicosapentaenoic acid (20:5n-3) and minimal arachidonic acid (20:4n-6) in marine fish oils leads to increased 20:5n-3/20:4n-6 in liver and plasma PL (19–22,24). The subsequent decrease in 20:4n-6 availability, substrate competition between n-6 and n-3 fatty acids for cyclooxygenase and/or a change in membrane fluidity may have possible functional implications to, for example, xenobiotic (25) or eicosanoid (26) metabolism, or immune function (27).

Piglets fed from birth with formula containing 34% of total fatty acids as 18:2n-6 and 0.8% of total fatty acids as 18:3n-3 had lower 22:6n-3, but higher 22:5n-6 in brain (5), synaptosomes (4) and retina (8) than piglets fed natural sow milk. The low CNS 22:6n-3 may be explained by an inadequate dietary supply of 18:3n-3 or limited ability to desaturate n-3, but not n-6, fatty acids. The increase in 22:5n-6 in the CNS lipids of these piglets (4,5,8) as well as n-3 fatty acid-deficient rodents (1,2), chicks (28) and non-human primates (7) provides evidence of Δ4 desaturase activity in the newborn.

The ability of various amounts of 18:3n-3 as compared to n-3 PUFA to support deposition of 22:6n-3 in brain at comparable levels to natural milk cannot be studied in humans. The piglet is a suitable animal model for this study because the timing of the perinatal brain growth spurt (29) and brain and milk lipid fatty acids (4) are similar to that of humans. The objective of this study was to determine if formula containing either 18:3n-3 as the only n-3 fatty acid, in amounts approximately four-fold greater than the total n-3 fatty acids in milk, or similar 18:3n-3 to milk plus C_{20} and C_{22} n-3 PUFA from fish oil can support equivalent deposition of 22:6n-3 in the CNS to sow milk. The quantity of fish oil used was similar to that added to formulas in clinical studies (10,11,19). The

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Abbreviations: ANOVA, analysis of variance; CNS, central nervous system; EPL, ethanolamine phospholipids; FO, fish oil; GLC, gas-liquid chromatography; HL, high α-linolenic acid; HPTLC, high-performance thin-layer chromatography; LL, low α-linolenic acid; PL, phospholipid; PUFA, polyunsaturated fatty acids; RBC, red blood cell; SM, sow milk; UI, unsaturation index.

four-fold greater 18:3n-3 was based on the work of Anderson *et al.* (28), which suggests 18:3n-3 was about four times less effective than 20:5n-3 and 22:6n-3 in supporting brain n-3 fatty acid deposition. The fatty acid analyses were restricted to synaptic plasma membrane and retina ethanolamine phospholipids (EPL), because these membranes are especially enriched in 22:6n-3 (30,31).

MATERIALS AND METHODS

Animals and diets. Male Yorkshire piglets of normal term gestation (116–118 days) were obtained from the University of British Columbia, Department of Animal Science and Pitt Ineffable Growers Ltd. (Pitt Meadows, B.C., Canada). Seven non-littermate piglets fed sow milk (SM) were kept on the farm and suckled from the sow. Six non-littermate piglets for each of the three formula groups were taken from the sow immediately *post partum*, prior to receiving colostrum. All animals were fed from birth to 15 days of age, representing at least two-thirds of the normal suckling period. Housing, feeding and provision of passive immunity with pig serum-derived immunoglobulin have been described previously (4). Spot heat lamps were attached to the cages to provide 24-hr light and heat for the first 7 days. An 0700-1900 hr light/1900–0700 hr dark cycle was maintained from 8–15 days to parallel lighting conditions of sow milk-fed piglets. The formula fats were blends of soy, coconut, corn, canola and/or high oleic acid sunflower oils, with or without marine oil. The fat contributed approximately 50% of dietary energy, similar to that provided by sow milk. The formulas supplied similar amounts of 18:2n-6, but with low (0.7% of the total fatty acids, LL) or high (3.9% of the total fatty acids, HL) 18:3n-3 or low 18:3n-3 with fish oil to provide 20:5n-3, 22:5n-3 and 22:6n-3 in amounts of 0.4, 0.1 and 0.3% of the total formula fatty acids, respectively (LL+FO) (Table 1). The formulas differed from sow milk in their higher percentage of fatty acids with C $\leqslant$ 14, lower percentage of 16:0 and absence of C_{20} and C_{22} n-6 PUFA. In contrast to LL+FO, LL and HL contained no detectable C_{20} and C_{22} n-3 PUFA. After opening, the liquid, read-to-feed formulas were kept at 4°C for no longer than 24 hr. The procedures used were approved by the University of British Columbia Animal Care Committee and conformed to the guidelines of the Canadian Council on Animal Care.

Sample collection and analyses. The non-fasted animals were sacrificed by intracardiac injection of 10 mL KCl (20 meq/10 mL) at 15 days of age. The cerebrum was immediately excised, weighed, minced and homogenized in 5 v/wt of 0.32 M sucrose/15mM Tris HCl (1mM EDTA, 1 mM $MgCl_2$ and 1.5 mM glutathione), pH 7.4. Synaptic plasma membranes were isolated by the method of Cruz and Gurd (33). Whole eyes were dissected within 15 min of death and kept on ice until retinas were removed with a gentle stream of ice-cold saline. All tissues were stored at −80°C until analysis.

Total lipids were extracted from all tissues according to Folch *et al.* (34). Lipid extracts were separated into PL classes on high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, v/v/v/v) (35) as the solvent system. Synaptic plasma membrane and retina EPL fractions, containing diacyl, alkylacyl and alkenylacyl glycerophosphoethanolamine, were collected. Fatty acid methyl esters were prepared, then separated and identified by gas-liquid chromatography (GLC) (4) using authentic fatty acid methyl ester standards (Supelco Inc., Bellefonte, PA; and Nu-Chek-Prep, Inc., Elysian, MN).

Statistical analysis. Differences in the mean levels of each fatty acid among the diet groups were investigated using one-way analysis of variance (ANOVA) (36). Formal tests of differences among diets utilized Fisher's least significant difference and were based on least squares means and standard errors calculated from ANOVA. Bonferroni corrections within a fatty acid were used to determine which of these differences were significant ($P < 0.01$). All tests for differences between means were two-sided. Outliers for each fatty acid detected by residual analysis methods were removed and the analysis was repeated. All calculations were performed using the General Linear Model procedure in the Number Cruncher Statistical System, version 5.01 (Kaysville, Utah).

TABLE 1

Fatty Acid Composition (% of total fatty acids) of Sow Milk and Formula[a]

Fatty acid	Sow milk[b] (SM)	Low α-linolenate (LL)	High α-linolenate (HL)	Low α-linolenate + fish oil (LL + FO)
14:0	4.0	32.2	28.3	34.6
16:0	30.5	8.1	7.9	7.8
18:0	4.4	3.4	3.8	3.3
18:1n-9	37.5	38.6	40.1	38.4
18:2n-6	11.1	15.6	16.6	15.5
18:3n-3	1.1	0.7	3.9	0.6
20:4n-6	0.5	nd[c]	nd	nd
20:5n-3	0.1	nd	nd	0.4
22:5n-3	0.2	nd	nd	0.1
22:6n-3	0.1	nd	nd	0.3

[a]The fatty acid composition of sow milk and formula was determined by direct 1-hr transesterification (100°C) with methanol/benzene (4:1, v/v) and acetyl chloride (ref. 32) and subsequent analysis by GLC.

[b]Values for sow milk are mean of 2 milk samples collected at 1- and 2-weeks lactation.

[c]nd, Not detected.

RESULTS AND DISCUSSION

Growth. The first 15 days of life are a period of exponential growth in the piglet. Body weight increased from about 1.2 kg to about 4.3 kg, while the total brain weight increased from about 27.9 g (5) to about 37.8 g over the 15 day study. This represents increases in body and brain weights of about 300% and 40%, respectively. Significant differences ($P < 0.05$) were not found among the four groups of piglets either in the final body (mean ± SEM: SM, 4.1 ± 0.4; LL, 4.3 ± 0.4; HL, 4.4 ± 0.3; and LL+FO, 4.3 ± 0.3 kg) or cerebrum (mean ± SEM: SM, 35.0 ± 1.0; LL, 34.1 ± 0.9; HL, 32.0 ± 0.6; and LL+FO, 33.3 ± 1.1 g) weight.

Effect of feeding low (LL) or high α-linolenate (HL) formula on tissue n-6 and n-3 fatty acids. The percentage of 22:5n-3, 22:6n-3, the ΣC_{20} and C_{22} n-3 PUFA and

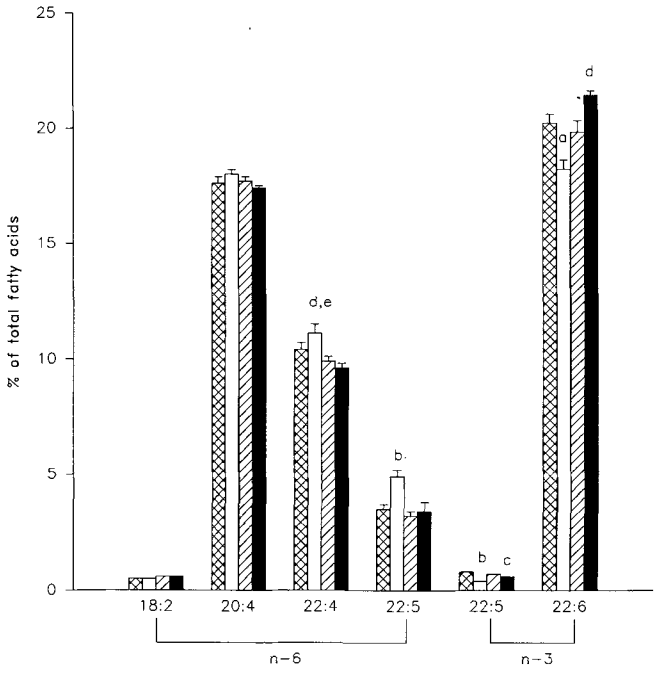

FIG. 1. Effect of dietary fatty acids on n-6 and n-3 fatty acids of synaptic plasma membrane ethanolamine phospholipids (EPL) of 15-day-old piglets fed: sow milk (SM), crossed bars; or formula containing 0.7% (LL, low linolenate), white bars; or 3.9% of total fatty acids as 18:3n-3 (HL, high linolenate), hatched bars; or 0.6% of total fatty acids as 18:3n-3 + 0.4, 0.1 and 0.3% of total fatty acids as 20:5n-3, 22:5n-3 and 22:6n-3, respectively (LL+FO, low linolenate + fish oil), black bars. Values are mean ± SEM (n=7, 6, 6, and 5, respectively). Values denoted with symbols represent statistically significant differences in the Fisher's least significant difference test, with Bonferroni correction, as follows: a, $P < 0.001$; and b, $P < 0.001$ *vs.* all other groups; c, $P < 0.001$ *vs.* SM; d, $P < 0.01$ *vs.* HL; and e, $P < 0.01$ *vs.* LL+FO.

22:6n-3/22:5n-6 were decreased and ΣC_{20} and C_{22} n-6 PUFA, specifically 22:5n-6, was increased in synaptic plasma membrane EPL of piglets fed LL as compared to SM (Fig. 1, Table 2). These changes are characteristic of neural tissues of rodents (1,2,37), non-human primates (7) and piglets (4,5,8) fed diets deficient in n-3 fatty acids. As in the synaptic plasma membrane, 22:5n-6 was increased and the ratio of 22:6n-3/22:5n-6 was decreased in the retina of LL compared to SM-fed piglets, although the percentage of 22:6n-3 was not significantly different (Table 2). In contrast, Hrboticky *et al.* (8) found significantly lower 22:6n-3 in the retina PE of piglets fed formula containing 34 and 0.8% of total fatty acids as 18:2n-6 and 18:3n-3, respectively. Possibly, competitive inhibition by the high levels of 18:2n-6 (18:2n-6/18:3n-3 ratio, 38:1) in the latter (8) compared to the LL formula (18:2n-6/18:3n-3 ratio, 22:1) may explain the difference in results. Despite the changes in individual C_{20} and C_{22} n-3 and n-6 fatty acids of piglets fed LL, the UI and ΣC_{20} and C_{22} PUFA within the structural PL of the neural tissues were similar among the groups (Table 2).

Levels of all fatty acids in the synaptic plasma membrane and retina EPL of piglets fed HL were equivalent to those of SM-fed piglets, with the exception of a lower retina 22:5n-3 in the HL group (Figs. 1 and 2, Table 2). The similar deposition of 22:6n-3 in neural tissues of HL and SM groups provides definitive evidence that, in this species, dietary 18:3n-3 can support synthesis and deposition of comparable amounts of 22:6n-3 in the absence of a dietary source of 22:6n-3. The higher amounts of 18:3n-3 in the HL formula may be necessary for adequate *de novo* synthesis of 22:6n-3 for deposition in structural membranes concomitant with probable oxidation of diet 18:3n-3 (38,39) to meet the high energy demands of the rapidly growing newborn. Whether or not a lower 18:2n-6/

TABLE 2

Comparison of Σ n-6, n-3 and Total C_{20} and C_{22} PUFA, Unsaturation Index (UI) and Ratio of % 22:6n-3/% 22:5n-6 in Synaptic Plasma Membrane and Retina Ethanolamine Phospholipids (EPL) in 15-Day-Old Piglets Fed Sow Milk or Formula[a]

	Synaptic plasma membrane EPL				Retina EPL			
Fatty acids	SM (n=7)	LL (n=6)	HL (n=6)	LL+FO (n=5)	SM (n=8)	LL (n=5)	HL (n=6)	LL+FO (n=5)
Σn-6 C_{20}-C_{22} PUFA[b]	31.4 ± 0.7	34.0 ± 0.7[c]	30.7 ± 0.5	30.4 ± 0.5	20.2 ± 0.6	22.2 ± 0.3[d]	18.6 ± 0.4	18.1 ± 0.5
Σn-3 C_{20}-C_{22} PUFA[e]	21.0 ± 0.4	18.6 ± 0.4[c]	20.5 ± 0.5	22.1 ± 0.2	33.1 ± 0.9	29.7 ± 0.7	32.1 ± 0.7	32.5 ± 1.0
Σ C_{20}-C_{22} PUFA	52.5 ± 1.1	52.6 ± 0.9	51.2 ± 0.9	52.5 ± 0.5	53.3 ± 1.1	51.9 ± 0.6	50.7 ± 0.4	50.6 ± 0.9
UI[f]	267 ± 5	265 ± 4	262 ± 4	270 ± 2	293 ± 6	284 ± 3	279 ± 5	282 ± 5
22:6n-3 / 22:5n-6	5.9 ± 0.3	3.8 ± 0.3[c]	6.3 ± 0.3	6.7 ± 0.7	22.0 ± 2.5	9.0 ± 0.4[c]	25.2 ± 1.6	28.4 ± 4.2

[a] See Table 1 for diet abbreviations.

[b] Σn-6 C_{20}-C_{22} PUFA, sum of 20:4n-6, 22:4n-6 and 22:5n-6.

[c] Significantly different ($P < 0.006$) from all other groups.

[d] Significantly different ($P < 0.0002$) from HL and from LL+FO.

[e] Σn-3 C_{20}-C_{22} PUFA, sum of 20:5n-3, 22:5n-3 and 22:6n-3.

[f] UI, unsaturation index = Σ (number of double bonds × % of total fatty acids).

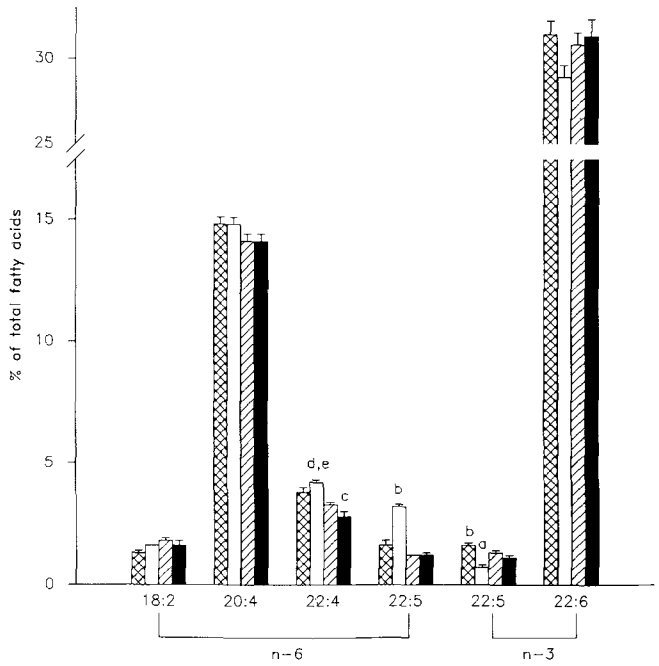

FIG. 2. Effect of dietary fatty acids on n-6 and n-3 fatty acids of retina ethanolamine phospholipids (EPL) of 15-day-old piglets fed sow milk (SM), crossed bars; or formula containing 0.7% (LL, low linolenate), white bars; 3.9% of total fatty acids as 18:3n-3 (HL, high linolenate), hatched bars; or 0.6% of total fatty acids as 18:3n-3 + 0.4, 0.1 and 0.3% of total fatty acids as 20:5n-3, 22:5n-3 and 22:6n-3, respectively (LL+FO, low linolenate + fish oil), black bars. Values are mean ± SEM (n=8, 5, 6 and 5, respectively). Values denoted with symbols represent statistically significant differences in the Fisher's least significant difference test, with Bonferroni correction, as follows: a, $P < 0.01$; and b, $P < 0.001$ *vs.* all other groups; c, $P < 0.001$ *vs.* SM; d, $P < 0.01$ *vs.* HL; and e, $P < 0.01$ *vs.* LL+FO.

18:3n-3 ratio than that in the LL formula (22:1) would facilitate assimilation of 22:6n-3 comparable to HL formula (18:2n-6/18:3n-3 ratio, 4:1) cannot be speculated from our results, but should be considered.

Diets supplying 0.44% of kcal as 18:3n-3 to newly hatched n-3 fatty acid deficient chicks have been shown to be inadequate to support normal levels of 22:6n-3 in brain and retina (28). This is in agreement with our results, showing that formula providing 0.3% of kcal as 18:3n-3 (LL) did not support normal 22:6n-3 deposition in the synaptic plasma membrane EPL. Recent studies have suggested the minimum dietary requirement for 18:3n-3 needed to achieve maximum levels of 22:6n-3 in rat total brain lipid is about 0.4% of kcal (1). Optimal amounts of 22:6n-3, however, do not appear to have been incorporated into the rat brain synaptosomes and retina in the latter studies until the diet 18:3n-3 intake was about 0.7% of kcal (1). This would allow a prediction that the HL formula, which provided 1.7% of kcal as 18:3n-3, would be an adequate source of n-3 fatty acids for the developing CNS. The results found support this prediction.

Effect of feeding formula containing low α-linolenate plus fish oil or high linolenate on tissue n-6 and n-3 fatty acids. Formulas containing low levels of fish oil to provide a diet source of 20:5n-3 and 22:6n-3 have been shown to be effective in maintaining plasma and RBC PL levels of 22:6n-3 in term (19) and preterm (10,11,24) infants similar to those found when human milk is fed. Further, addition of marine oils to diets containing limited 18:3n-3 are effective in supplying 22:6n-3 to developing piglet (20,21) and rodent (22,23) brain. Feeding the LL+FO formula resulted in similar ΣC_{20} and C_{22} n-3 PUFA and 22:6n-3, but lower 22:5n-3, in the synaptic plasma membrane and retina EPL to SM-fed piglets (Figs. 1 and 2, Table 2). The levels of 22:6n-3 were significantly higher in the synaptic plasma membrane EPL of the LL+FO group than in the HL group, however, values were not significantly different from those of the SM-fed piglets. Yamamoto *et al.* (2) reported similar levels of 22:6n-3 in brain between rats fed 8% of diet kcal as 18:3n-3 and rats fed 0.5% plus 0.3% of kcal as 18:3n-3 and C_{20} and C_{22} n-3 PUFA, respectively. In contrast, feeding diets containing fish oil to provide 5-6% of kcal as C_{20} and C_{22} n-3 PUFA resulted in a higher percentage of 22:6n-3 in cerebral cortex of monkeys (3) and brain microsomes of rats (40) than when a control diet providing 1-2% of kcal as 18:3n-3 was fed. Based on estimates of dietary requirements for 18:3n-3 of less than 1.0% of kcal (1), it seems possible that the elevation of C_{20} and C_{22} n-3 PUFA seen with fish oil supplementation at more than 5% of kcal reflects pharmacological changes rather than optimal physiology.

Our results indicate that 1.7% of dietary kcal as 18:3n-3 is as effective as 0.4% of kcal as C_{20} and C_{22} n-3 PUFA in achieving levels of 22:6n-3 in the growing piglet synaptic plasma membrane and retina. Thus, as suggested by others (28), our results show that dietary 18:3n-3 is at least 24% as efficient as C_{20} and C_{22} n-3 PUFA as a source of CNS 22:6n-3 in the newborn piglet.

The synaptic plasma membrane and retina EPL fatty acids contained less than 0.1% of total fatty acids as 20:5n-3, even in piglets fed HL or LL+FO formula (data not shown). Levels of 20:4n-6 were not altered in these CNS tissues by formula feeding (Figs. 1 and 2). Similarly, lack of accumulation of 20:5n-3 (3,20,21,40) and maintenance of 20:4n-6 in brain (20,21,37) have been reported following feeding with up to 8% of kcal as 18:3n-3 (37) or 0.4 to 6% of kcal as C_{20} and C_{22} n-3 PUFA (3,20,21,40).

The only significant difference found in the synaptic plasma membrane and retina EPL n-6 fatty acids among the SM, HL and LL+FO piglet groups was a lower 22:4n-6 in the retina EPL of LL+FO compared to SM-fed piglets (Fig. 2, Table 2). Whether or not this is related to the fish oil and has any physiological significance is unknown. It is known, however, that 22:4n-6 may be chain shortened (41), thus possibly contributing to the maintenance of membrane PL 20:4n-6.

In summary, the results of this study demonstrate that the provision of 1.7% of kcal as 18:3n-3 in formula containing 8% of kcal as 18:2n-6 is adequate for normal C_{20} and C_{22} n-3 and n-6 PUFA deposition in the CNS of term gestation piglets. The relatively high formula 18:3n-3, giving an 18:2n-6/18:3n-3 ratio of 4:1, did not result in any reduction in the normally high levels of C_{20} and C_{22} n-6 PUFA in CNS tissues examined. Small amounts (0.4% of kcal) of C_{20} and C_{22} n-3 PUFA were also effective in supporting 22:6n-3 deposition in the synaptic plasma membrane and retina and did not lead to accumulation of 20:5n-3 or loss of 20:4n-6.

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Does a Threshold for the Effect of Dietary Omega-3 Fatty Acids on the Fatty Acid Composition of Nuclear Envelope Phospholipids Exist?

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Existence of a dietary maximal level or threshold for incorporation of ω3 fatty acids into membrane phospholipids is of interest as it may further define understanding of the dietary requirement for ω3 fatty acids. To test whether feeding increasing levels of dietary ω3 fatty acids continues to increase membrane ω3 fatty acid content, weanling rats were fed a nutritionally adequate semipurified diet which provided increasing amounts of C_{20} and C_{22} ω3 fatty acids, such as 20:5ω3 and 22:6ω3. Dietary 20:5ω3 and 22:6ω3 were provided by substituting a purified shark oil concentrate of high 22:6ω3 content for safflower oil high in 18:2ω6. After four weeks of feeding, nuclear envelopes from four animals in each diet group were prepared, lipid was extracted and phospholipids separated. Arachidonic acid content in membrane phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine was significantly reduced by feeding increased dietary levels of ω3 fatty acids. Decline of 20:4ω6 level in phospholipid tended to stabilize when the dietary content of total ω3 fatty acids reached 4–5% of total fatty acids. Above this level, dietary ω3 fatty acids did not result in a further decrease in membrane content of 20:4ω6. Increase in membrane phospholipid content of 20:5ω3 occurred as the dietary intake of ω3 fatty acids increased from 1.1% to 5% of total fatty acids. A dietary ω3 fatty acid level of 2.2–3% was sufficient to result in maximum incorporation of 22:6ω3 into membrane phosphatidylcholine and phosphatidylethanolamine, but not into phosphatidylinositol or phosphatidylserine.
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The role of dietary omega-3 fatty acids has been studied in a wide variety of membranes (reviewed in ref. 1). When eicosapentaenoic acid (20:5ω3) is fed to rats, 20:5ω3 and docosahexaenoic acid (22:6ω3) levels increase in membrane phospholipids. It is not clear whether 22:6ω3 increases in membrane solely as a consequence of *in vivo* conversion of 20:5ω3 to 22:6ω3 or because most sources of 20:5ω3 also contain some 22:6ω3. Thus, relatively little is known about the incorporation of dietary docosahexaenoic acid into membrane lipids. Recent experiments (2) have used various dietary levels of 18:3ω3 to discern a dietary threshold level of 0.4 to 2.4% of energy to produce significant changes in brain membrane 22:6ω3 and 22:5ω6 levels. Not only is minimum dietary levels of ω3 fatty acid required for incorporation of ω3 fatty acids into membrane lipid to affect integral membrane functions clearly of interest, it is central to current debate over the optimal fatty acid composition of infant formulas, and may help to establish dietary requirements for ω3 fatty acids.

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Feeding diets high or low in polyunsaturated to saturated fats induces differences in fatty acid composition of the nuclear envelope phospholipid in the liver, altering functions involved in hormone binding (3), mRNA synthesis and nucleocytoplasmic exchange of macromolecules (4). Thus, diet-induced change in the composition of this membrane alters functions central to the activity of the cell.

The present study used a feeding model to determine whether gradual increase in dietary ω3 fatty acids, primarily 22:6ω3 and 20:5ω3, results in a continuous increase in the content of 20:5ω3 and 22:6ω3 in nuclear envelope phospholipids. We hypothesized that if a dietary threshold exists it would result in a maximum level of incorporation of ω3 fatty acid in membrane phospholipids.

MATERIALS AND METHODS

Animals and diets. Weanling Sprague-Dawley strain rats were fed one of eight nutritionally adequate semipurified diets that provided different levels of C_{20} and C_{22} ω3 fatty acids, such as 20:5ω3 and 22:6ω3 (Table 1) (5). The 18:3ω3 level of the diets was kept constant (Table 2). Dietary 20:5ω3 and 22:6ω3 were provided by substituting a purified shark oil concentrate of high 22:6ω3 content for safflower oil high in 18:2ω6 (Table 1). Data are expressed as a function of increasing dietary intake of ω3 fatty acid.

After four weeks of feeding, four animals from each diet were sacrificed by cervical dislocation. Livers were quickly removed and placed on ice. Subsequent procedures were done at 4°C unless stated otherwise.

Preparation of nuclear envelope. Nuclear envelopes were prepared from the purified nuclear pellet by subjecting nuclei to deoxyribonuclease (Sigma Chemical Co., St. Louis, MO) digestion and were purified by layering on a discontinuous sucrose gradient (6). Succinic dehydrogenase activity could not be detected in these preparations, indicating that membrane preparations were free of mitochondrial contamination. Contamination by lysosomes, plasma membranes and endoplasmic reticulum was also very low. Electron microscopic examination indicated that the nuclear envelope preparations were intact, with inner and outer membranes well preserved.

Extraction and analysis of lipids. Nuclear membrane lipids were extracted and phospholipids were separated (7,8). Lipids were identified and recovered; fatty acid methyl esters were prepared using boron trifluoride/methanol reagent (9). Saturated and unsaturated fatty acids of 14–24 carbons in chain length were analyzed by automated capillary gas-liquid chromatography using a fused silica column (Scientific Gas Engineering, Victoria, Australia, BP-20 column, 25 m × .25 mm ID) (10). Fatty acids analyzed included all saturated and unsaturated fatty acids of 14–24 carbons in chain length. Only major ω6 and ω3 fatty acids are listed.

TABLE 1

Composition of the Diets Fed

Ingredient	Low P/S[a] diet 1	Diet containing fish oil 2	3	4	5	6	7	8
				(g/kg of Total diet)				
Basal[b]	800	800	800	800	800	800	800	800
Safflower oil	27.2	27.5	26.1	24.0	20.8	18.7	16.6	13.4
Hydrogenated beef tallow	169	172.0	157.2	155.1	151.9	149.8	147.7	137.6
Linseed oil	4	4.2	4.2	4.2	4.2	4.2	4.2	4
Fish oil	—	6.3	12.6	16.8	23.1	27.3	31.5	35.0
ω3 Fatty acid content	1.1	2.0	3.0	3.7	4.8	5.4	6.0	7.3

[a] P/S represents polyunsaturated to saturated fatty acid ratio.

[b] The basal diet provided (g/kg of total diet): 270 g casein, 378 g starch, 80 g cellulose, 10 g vitamin mix, 50 g mineral mix, 2.75 g choline, 6.25 g inositol and 2.5 g L-methionine (6).

TABLE 2

Fatty Acid Composition of the Diets[a]

Fatty acids	Low P/S diet 1	Fish oil diet 2	3	4	5	6	7	8
				(% w/w)				
16:0	24.1	24.6	24.0	24.0	24.1	24.1	24.8	25.0
18:0	53.2	51.8	51.4	49.1	49.0	48.9	48.6	45.6
18:1ω9	4.8	5.2	5.6	10.0	9.9	5.6	5.7	9.3
18:2ω6	10.5	10.4	9.9	9.5	8.2	7.5	6.9	6.6
18:3ω3	1.1	1.1	1.2	1.2	1.3	1.4	1.2	1.3
20:5ω3	—	0.19	0.38	0.54	0.79	0.83	0.96	1.2
22:6ω3	—	0.8	1.5	2.0	2.7	3.3	3.7	4.4
Σω3[b]	1.1	2.0	3.0	3.7	4.7	5.4	6.0	7.3
P/S ratio	1.96	0.15	0.16	0.17	0.18	0.18	0.18	0.21

[a] All dietary saturated and unsaturated fatty acids of C_{14} to C_{24} in chain length were analyzed; however, only major constituents are listed.

[b] Σω3, Total ω3 fatty acids.

Assays. Protein content was determined (11). Marker enzymes were measured (12) to determine the purity of nuclear envelope preparations. Data were assessed by regression procedures to determine the best fit line. Values (n ± SD) for each diet treatment are illustrated with the best fit line.

RESULTS AND DISCUSSION

Feeding diets of increasing ω3 fatty acid content altered the fatty acid composition of the nuclear envelope (Figs. 1 and 2). The degree of change which occurred in membrane composition was different for individual essential fatty acids. For example, increasing dietary content of ω3 fatty acids resulted in a relatively small change in the content of 18:2ω6 in phosphatidylcholine (Fig. 1) and phosphatidylinositol (Fig. 2). However, arachidonic acid content in membrane phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine was significantly reduced by feeding increasing levels of ω3 fatty acid. Decline of the 20:4ω6 level in these phospholipids tended to reach a minimum level when the dietary content of total ω3 fatty acids reached 4–5% of total fatty acids (Figs. 1 and 2). Feeding levels beyond this point did not result in further decrease in membrane phospholipid content of 20:4ω6.

Increase in membrane phospholipid content of 20:5ω3 occurred as the dietary intake of ω3 fatty acids was increased from 1.1% to 5% of total fatty acids. Further increase in dietary ω3 fatty acid level did not result in increased incorporation of 20:5ω3 into phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol or phosphatidylserine. Dietary levels of 2.2–3% of total ω3 fatty acid were sufficient to result in maximum incorporation of 22:6ω3 into membrane phosphatidylcholine and phosphatidylethanolamine. For phosphatidylinositol, increase in 22:6ω3 content occurred as the dietary content of ω3 fatty acids, and thus dietary 22:6ω3 increased. It is clear that feeding a high level of 22:6ω3 alters membrane phospholipid content of 20:4ω6 and 20:5ω3 most effectively over the range of 1.1–5% of dietary fatty acid fed. By estimating the amount of dietary 20:5ω3 intake (see

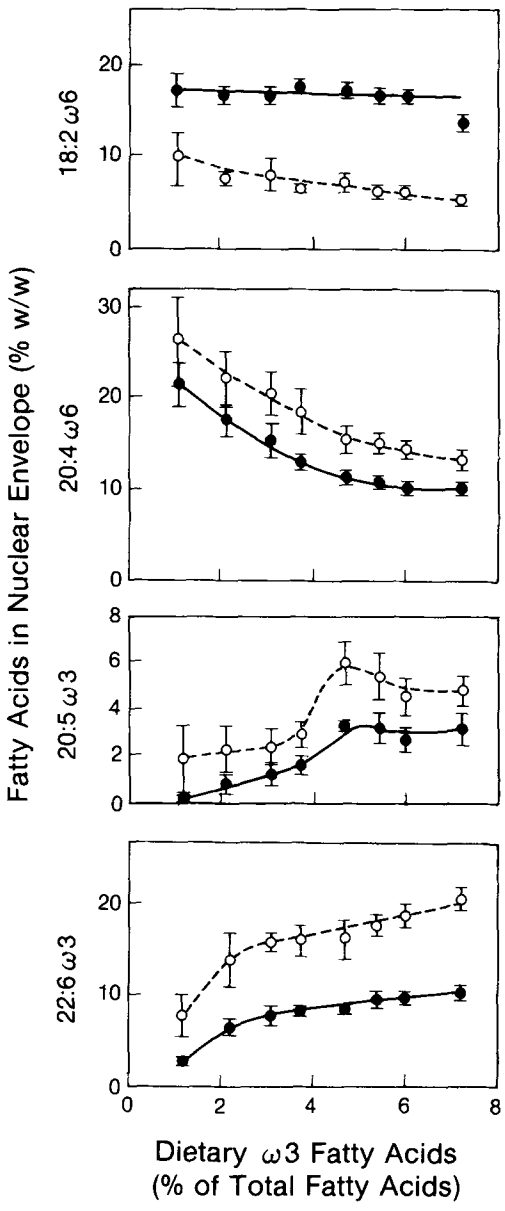

FIG. 1. Effect of increasing levels of dietary ω3 fatty acid on the fatty acid composition of the phosphatidylcholine (●) and phosphatidylethanolamine (○) fractions of rat liver nuclear envelope. The dietary fatty acid composition for each point illustrated can be determined from Table 2. The point closest to the intersect of the x and y axes is diet 1, with diets 2-8 following (Figs. 1 and 2).

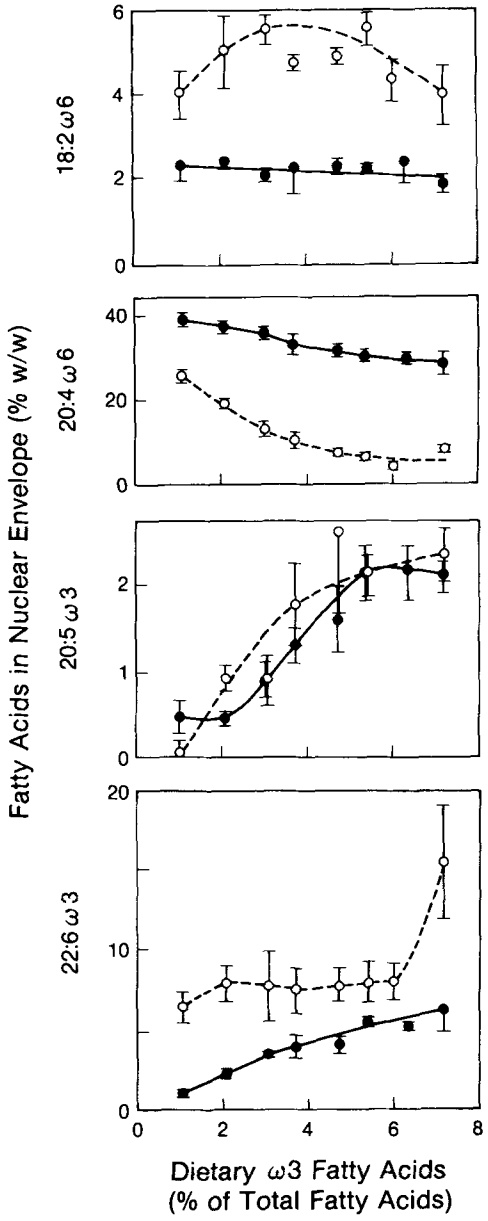

FIG. 2. Effect of increasing concentrations of dietary ω3 fatty acids on the fatty acid composition of the phosphatidylinositol (●) and phosphatidylserine (○) fractions of rat liver nuclear envelope.

scale in Figs. 1 and 2), it is apparent that a similar dietary intake of 20:5ω3 (0.79 to 0.83% of fatty acid) and 22:6ω3 (0.75-1.5% of fatty acid) is reflected in a plateau in membrane phosphatidylcholine and phosphatidylethanolamine content of 20:5ω3 and 22:6ω3 (Fig. 2). This suggests that increase in membrane content of 20:5ω3 and 22:6ω3 is primarily in response to the dietary level of these fatty acids fed and is not primarily attributable to synthesis from 18:3ω3 in the diet.

It is clear from the current study that a dietary threshold for the effect of dietary 20:5ω3 and 22:6ω3 on the fatty acid composition of nuclear envelope membrane phosphatidylcholine and phosphatidylethanolamine composition exists. The threshold level for both fatty acids is apparently similar and suggests that a maximal effect for dietary 20:5ω3 and 22:6ω3 on membrane phosphatidylcholine and phosphatidylethanolamine fatty acid composition occurs at a relatively low dietary concentration consistent with that observed for brain when the only ω3 fatty acid fed was 18:3ω3 (2).

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The Effects of Dietary n-3/n-6 Ratio on Brain Development in the Mouse: A Dose Response Study with Long-Chain n-3 Fatty Acids

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This study examines the effects of the ratio of n-3/n-6 fatty acids (FA) on brain development in mice when long-chain n-3 FA are supplied in the diet. From conception until 12 days after birth, B6D2F$_1$ mice were fed liquid diets, each providing 10% of energy from olive oil, and a further 10% from different combinations of free FA concentrates derived from safflower oil (18:2n-6), and fish oil (20:5n-3 and 22:6n-3). The range of dietary n-3/n-6 ratios was 0, 0.25, 0.5, 1.0, 2.0 and 4.0, with an n-6 content of greater than 1.5% of energy in all diets, and similar levels of total polyunsaturated fatty acids (PUFA). In an additional group of ratio 0.5, 18:2n-6 was partially replaced by its Δ6 desaturation product, 18:3n-6. Biochemical analyses were conducted on 12-day-old pup brains, as well as on samples of maternal milk. No obvious effects on overall pup growth and development were observed, apart from a smaller litter size at ratio 1. Co-variance analysis indicated that increasing the n-3/n-6 ratio was associated with slightly smaller brains, relative to body weight. We found that 18:2n-6 and 20:5n-3 were the predominant n-6 and n-3 FA in the milk; in the brain these were 20:4n-6 and 22:6n-3, respectively. Increasing dietary n-3/n-6 ratios generally resulted in an increase in n-3 FA, with a corresponding decrease in n-6 FA. The n-3/n-6 ratio of the milk lipids showed a strong linear relationship with the diet, but in the brain the rate of increase tended to decrease beyond 0.5 (phosphatidylcholine, PC) and 0.25 (phosphatidylethanolamine, PE), such that there was a significant quadratic contribution to the relationship. The partial replacement of dietary 18:2n-6 with 18:3n-6 raised levels of 20:4n-6 in milk, brain PC, and brain PE. These results indicate that the n-3/n-6 ratio of the phospholipids in the developing mouse brain responds maximally to maternal dietary long-chain n-3/n-6 ratios of between 0.25 and 0.5.
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The membrane phospholipids of the central nervous system contain high levels of polyunsaturated fatty acids (PUFA), particularly arachidonic acid, 20:4n-6, and docosahexaenoic acid, 22:6n-3 (1). It is known that 22:6n-3, occurs at high concentrations in synaptic membranes (2) and in photoreceptor cells in the retina (3,4). These long-chain PUFA accrue rapidly in the brain during the prenatal and suckling periods (5–7), and are formed from their respective dietary precursors, 18:2n-6 and 18:3n-3, by the same series of desaturation and elongation reactions, with n-3 having the competitive advantage over n-6 compounds *in vitro* (8). Both fetal liver and brain have the metabolic capacity to synthesize long-chain PUFA (9,10). There is recent evidence suggesting that 18:3n-3 is converted in fetal and pup liver to 22:6n-3, which is then secreted into blood as lipoproteins, and incorporated selectively into nervous tissue (11). Studies in both developing rat and chick brain have demonstrated preferential uptake of 22:6n-3 over its precursors (12,13). Dose-response studies conducted by varying the amount of dietary 18:3n-3 during the developmental period (14), and in weanling animals (15–17), showed a decrease in brain 22:5n-6 and an increase in brain 22:6n-3 with increasing dietary 18:3n-3. Much of this effect appeared to be in comparison with an n-3 deficient group, with smaller differences at the higher dosage levels, suggesting regulatory limits. The relationship between brain FA composition and dietary long-chain n-3 FA may be different from that seen with dietary 18:3n-3. Feeding of fish oil to adult rats resulted in a rapid increase in levels of 22:5n-3 and 22:6n-3, as well as 20:5n-3 (which is usually present in brain only in trace amounts), with corresponding decreases in 22:5n-6, as well as 20:4n-6, suggesting that the brain may be vulnerable to an excess of long-chain n-3 PUFA (18,19). The developing brain, because of its affinity for long-chain n-3, may be particularly susceptible to such effects. There is particular concern that decreases in 20:4n-6 may be associated with adverse effects (20). Thus the present study investigated the relationship between the dietary long-chain n-3/n-6 ratio and the FA composition of the brain in developing mice. The n-3 FA were provided as the long-chain compounds found in fish oil, predominantly 20:5n-3 and 22:6n-3, and using ratios of 0, 0.25, 0.5, 1.0, 2.0 and 4.0. While the 0 group was clearly an n-3 deficient group, the values above 0.25 considerably exceeded current dietary recommendations (21), with the intent of challenging the regulatory capacity of the system. The study included two groups of ratio 0.5, one as part of the dose-response series where the n-6 was provided as 18:2n-6, and the other where a portion of the 18:2n-6 was replaced by 18:3n-6. Previous work comparing labelled dietary 18:3n-6 with 18:2n-6 showed increased incorporation into brain 20:4n-6 with 18:3n-6 (22). Therefore comparison of these two groups addressed whether levels of 20:4n-6 in the brain would increase in animals fed 18:3n-6 relative to those receiving the same amount of long-chain n-3, but only 18:2n-6 in the diet. The dietary treatments were imposed throughout gestation and lactation. The FA composition of milk lipids and pup brain phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions was determined on day 32 postconception (12 days after birth); other measurements included pup body and brain growth, as well as eye-opening score.

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Abbreviations: ANOVA, analysis of variance; EFA, essential fatty acids; FA, fatty acids; GLC, gas-liquid chromatography; GLM, general linear model; MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; SAS, Statistical Analysis Systems; SFA, saturated fatty acids.

MATERIALS AND METHODS

Animals. Parents were 28- to 32-week-old B6D2F$_1$ hybrid mice purchased from Charles River Breeding Laboratories (St. Constant, P.Q., Canada). They were maintained under a reversed 12 hr light:12 hr dark schedule at 22 ± 1°C, with tap water and lab chow (PMI Lab Diet—formerly Purina—# 5001, St. Louis, MO) available *ad libitum* until breeding. The animals were group-housed in standard plastic cages containing Beta-Chip hardwood bedding (Northeastern Products Corp., Warrensburg, NY) and toilet tissue for nesting material.

Diets. Maternal animals were fed one of six liquid diets, each of which provided 20% of the calories from oil, but which varied in their n-3/n-6 ratio. The diet, as used in our previous studies (23–25), was specifically formulated for our use by BioServ, ref. # F2187 (Frenchtown, NJ). This provided 1 kcal/mL, with 20% of the calories from protein (fat-free casein), 20% from oil, 60% from carbohydrate (maltose-dextrin), supplemented with minerals and vitamins. The dietary oil mixtures were prepared in our laboratory from olive oil (13% 18:2n-6 as triglyceride), and free fatty acid concentrates, Safflower 70, derived from safflower oil and containing 69.7% 18:2n-6, and EPA 50, derived from fish oil concentrate and containing 41.2% 20:5n-3 and 6.1% 22:6n-3 (Callanish Ltd., Breasclete, Isle of Lewis, U.K.). The use of these free fatty acid concentrates in the formulation of the diets allowed us to provide high n-3/n-6 ratios (0.0, 0.25, 0.5, 1.0, 2.0, and 4.0), while ensuring similar levels of PUFA (approximately 8% of total dietary energy) (Table 1). All groups received sufficient n-6 FA (>1.5% of total dietary energy), such that we were studying only the effect of increasing n-3, and not that of n-6 deficiency. The n-6 was supplied as 18:2n-6, and the n-3 predominantly as 20:5n-3 and 22:6n-3. This design therefore addressed the effects of increasing the dietary n-3/n-6 ratio ((20:5n-3 + 22:6n-3)/18:2n-6) while keeping the total amount of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) relatively constant; thus, as n-3 increased, n-6 decreased. The alternative of increasing n-3 while keeping n-6 constant would have had the problem of causing these other factors to vary concomitantly. A similar dietary formulation has been used in a recently published study of varying dietary n-6/n-3 ratio on the brain fatty acid (FA) composition in weanling rats (27). An additional group was fed a diet with a n-3/n-6 ratio of 0.5 and the same amount of EPA 50 as the 0.5 group. In this group, 18:2n-6 was replaced partially by 18:3n-6 (GLA 70, containing 19% 18:2n-6 and 68.7% 18:3n-6). Additionally, α-tocopherol was added to the oil mixtures, such that the prepared liquid diet contained 150 i.u. per liter.

Breeding protocol. Animals were mated daily, and checked for the presence of copulatory plugs after 7 hr. Pregnant dams were assigned randomly to each dietary condition on day 0, and all subsequent days were designated as "days post-conception." Dams were fed daily preweighed amounts of the liquid diet, based on 0.65 kcal/g body wt/day, until day 20. Fresh diet was prepared every two days, and diets and dietary oils were stored in the refrigerator under nitrogen at all times. Birth occurred on day 19 or 20, and all dams and litters were weighed on

TABLE 1

Selected Fatty Acid Composition of Dietary Oil Mixtures[a]

	0	0.25	0.5	1.0	2.0	4.0	0.5[b]
% Energy							
Olive oil	50.00	50.00	50.00	50.00	50.00	40.00	56.7
Safflower 70	50.00	35.70	26.70	16.00	5.85	1.00	
EPA 50		14.30	23.30	34.00	44.15	59.00	23.3
GLA 70							20.0
% Fatty acids							
16:0	10.8	9.8	9.1	8.4	7.7	5.9	8.3
16:1	0.7	2.5	3.6	4.3	6.3	8.0	0.8
18:0	2.4	3.1	3.5	4.0	4.5	5.2	3.8
18:1n-9	39.6	38.1	37.2	36.0	35.0	28.2	37.9
18:1n-7		0.3	0.5	0.7	0.9	1.2	0.5
18:2n-6	41.4	31.7	25.7	18.5	11.6	7.2	11.7
18:3n-6		0.2	0.3	0.4	0.5	0.7	14.0
20:4n-6		0.1	0.2	0.2	0.3	0.4	0.2
20:5n-3		5.9	9.6	14.0	18.2	24.3	9.6
22:6n-3		0.9	1.4	2.1	2.7	3.6	1.4
Other n-3[c]		1.2	2.1	3.1	4.0	5.4	2.1
Total SFA	13.2	12.9	12.6	12.4	12.2	11.1	12.1
MUFA	40.3	40.9	41.3	41.0	42.2	37.4	39.2
PUFA	41.4	40.0	39.0	38.3	37.3	41.6	39.2
n-3		8.0	13.1	19.2	24.9	33.3	13.1
n-6	41.4	32.0	26.2	19.1	12.4	8.3	25.9

[a] Calculations are based on GLC analysis of component oils (olive oil, 13% n-6; safflower 70, 70% n-6; EPA 50, 4% n-6, 56% n-3; GLA 70, 88% n-6).

[b] In this group the dietary 18:2n-6 was replaced partially by 18:3n-6.

[c] 18:4n-3, 20:4n-3, 22:5n-3.

day 20. At this time, litters were culled to six (three of each sex). From day 20 until day 32 post-conception, when the pups were tested, lactating mothers were fed the appropriate liquid diet *ad libitum* at 1.5 × strength (daily intake = 0.975 kcal/g). Differences in food intake among the groups were negligible. On day 32, two pups of each sex from each litter were assessed for eye-opening on a scale from 0 to 1 (detailed criteria available on request). One male and one female from each litter were then anaesthetized with Halothane and decapitated. The brains were extracted and trimmed by removal of the olfactory bulbs anteriorly and 2 mm below the medulla posteriorly. The two brains from each litter were pooled, weighed within two minutes to the nearest 0.1 mg, and frozen at −50°C until analysis of fatty acid composition. The hearts, livers and kidneys were also extracted, pooled and stored; these data are the subject of a separate report (26). The remaining pups were separated from the dams overnight, and on day 33 the dams were milked, following an adaptation of the procedure described by Mills *et al.* (27). The milk was also stored at −50°C. All tissue was coded so that biochemical analysis was done independently of knowledge of the treatment group.

Brain lipids. The homogenized brains were extracted according to a modified method of Bligh and Dyer (28), using chloroform/methanol (1:1, v/v), in the presence of 0.02% BHT (w/v). After separation and drying under nitrogen, the total lipids were fractionated by thin-layer chromatography, using silica gel plates (Analtech GF) and a chloroform/methanol/acetic acid/water (50/30/4/2, by vol) solvent system. The fatty acids of the resulting phospholipid fractions were methylated with 14% boron trifluoride in methanol, and analyzed on a gas chromatograph (Perkin-Elmer 8420, Norwalk, CT) equipped with a flame-ionization detector and a 15 m × 0.32 m (i.d.) capillary column (Supelco Wax 10, Bellefonte, PA). The temperature program for gas-liquid chromatography (GLC) consisted of a 2-min hold at 160°C, followed by a 2°/min increase to 190°C. After 10 min at 190°C the temperature was increased at 5°/min to 220°C, followed by a 2-min hold at 220°C. Fatty acids were identified by comparison of their retention times with those of authentic standards.

The concentration of total phospholipids, and the percent distribution of phospholipid subclasses were measured by high-performance liquid chromatography (System Gold, Beckman Canada, Mississauga, Ontario, Canada) using a Si Ultrasphere column (5 microns, 4.6 mm i.d. × 250 mm, Beckman) and a mass detector (ACS 750/14, Applied Chromatography Systems Ltd., Lutons, Bds., U.K.) as described previously (29).

Statistical analyses. The maternal and pup growth data were analyzed using the general linear model (GLM) provided by the Statistical Analysis System (SAS) to do analysis of variance (ANOVA). Individual group means were compared using Tukey's t-test; least squares means resulting from covariance analysis were compared using t-tests; the alpha level was set at 0.05. In the absence of a treatment by sex interaction, the litter mean score, collapsed across sex, was used as the unit of analysis for the data on pup growth and development. As the fatty acid determinations were conducted on the pooled brains from a litter, the litter also represents the unit of analysis for these measures. Regression analyses described the nature of the dose-response relationship between the dietary n-3/n-6 ratio and the n-3/n-6 ratio of milk and brain lipids. A linear model was used initially, and, when this proved significant, it was subsequently determined whether the inclusion of a quadratic component made a significant additional contribution. The sample sizes are shown in the respective tables.

RESULTS

Maternal variables and pup growth and development. Maternal weight gain did not differ among the groups during pregnancy, but litter size, as reflected by the number of live pups on day 20, was significantly different, $F(5,45) = 3.73$, $p < 0.01$, with ratio 1.0 having smaller litters (mean (pups) ±SEM: 5.3 ± 0.9) than ratio 0.25 (9.0 ± 0.5) and ratio 2.0 (9.1 ± 0.8). Neither pup weight on day 20 and day 32, nor eye-opening score, differed significantly. Brain weight showed a trend toward decreasing as the n-3/n-6 ratio increased, particularly at ratio 2.0. This was confirmed by a covariance analysis, where the least squares means of brain weight adjusted for body weight indicated a significant treatment effect, $F(5,41) = 3.08$, $p < 0.02$, with ratio 0.25 (mean (g) ± SEM: 0.347 ± 0.003), 1.0 (0.345 ± 0.003), and 2.0 (0.342 ± 0.003) being significantly lower than ratio 0 (0.356 ± 0.003. Ratio 4.0 (0.348 ± 0.003) was marginally lower than ratio 0, $p < 0.08$. Ratio 0.5 (3.353 ± 0.003) did not differ from any other group.

Lipid analysis. The n-3/n-6 ratio provides a summary of the outcome of the dietary treatment. There was a clear treatment effect on the milk lipids, $F(5,34) = 841.34$, $p < 0.0001$, brain phosphatidylcholine (PC), $F(5,40) = 191.6$, $p < 0.0001$, and brain phosphatidylethanolamine (PE), $F(5,41) = 164.9$, $p < 0.0001$. As is shown in Figure 1, the relationship between the n-3/n-6 ratio of the maternal diet and that of the milk was represented by a linear dose-response model. The pattern in the brain phospholipids

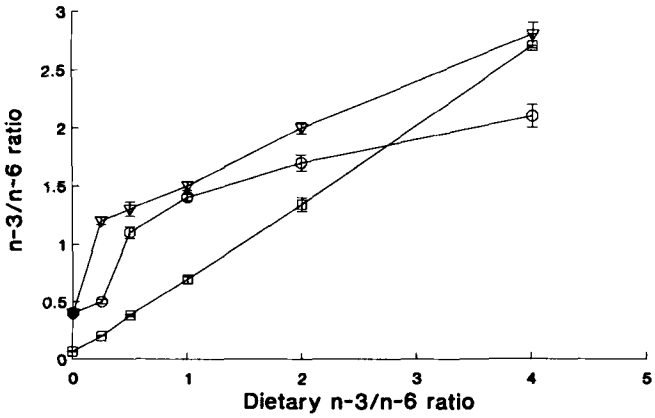

FIG. 1. Effects of maternal dietary n-3/n-6 ratio (x) on the n-3/n-6 ratio (y) of milk lipids (□) and pup brain phosphatidylcholine (PC, ○) and phosphatidylethanolamine (PE, ▽). Pregnant mice were fed diets of increasing n-3/n-6 ratio from conception to day 32 post-conception (12 days after birth). The values represent the group mean ±SEM. Regression analysis shows a linear relationship of the treatment with milk lipids ($y = 0.04 + 0.67x$, $r^2 = 0.99$) whereas in brain the relationship includes a quadratic component (PC: $y = 0.41 + 1.05x - 0.16x^2$, $r^2 = 0.90$; PE: $y = 0.70 + 0.88x - 0.09x^2$, $r^2 = 0.90$. In PC, all groups differ except 0.25 and 0.0: in PE all groups differ except 0.5 which does not differ from 0.25 and 1.0.

was somewhat different. In both the PC and PE fractions there was an initial steep increase, followed by increments which were smaller in magnitude, although still significantly different from each other. Thus the relationship with the dietary n-3/n-6 ratio included a significant quadratic component. In PC, all groups differed except 0.25 and 0.0; in PE all groups differed except 0.5 which did not differ from 0.25 and 1.0.

The values for individual FA are given in Tables 2, 3, and 4. From these it is clear that in both PC and PE the increase in the n-3/n-6 ratio was due to an increase in n-3, with a reciprocal decrease in n-6. In the milk lipids, 18:2n-6 and 20:5n-3 were the major FA. Their elongation products, 20:4n-6 and 22:6n-3, were the predominant n-6 and n-3 compounds, respectively, in the brain. Very little 18:2n-6 was observed in the brain, and 20:5n-3 was seen only in any appreciable amounts (>1%) in the PE fraction of the groups receiving n-3/n-6 ratios of 2 and 4. Conversely, 22:5n-6 was seen only at ratio 0 in both PC and PE. In contrast to other n-6 FA, levels of 20:3n-6 increased as the

TABLE 2

Effects of Maternal Dietary n-3/n-6 Ratio on Selected Fatty Acid Composition of Milk Lipids on Day 33 Postconception[a]

n-3/n-6 Ratio	0.0 n = 9	0.25 n = 7	0.5 n = 6	1.0 n = 7	2.0 n = 6	4.0 n = 6	0.5[b] n = 7
14:0	13.3 ± 0.3	14.0 ± 0.4	14.6 ± 0.3	13.6 ± 0.5	14.4 ± 0.5	15.5 ± 0.4	14.9 ± 0.3
16:0	21.0 ± 0.4	22.1 ± 0.6	22.9 ± 0.6	22.5 ± 0.8	22.0 ± 0.6	21.6 ± 1.1	19.8 ± 3.2
16:1	1.5 ± 0.07	1.9 ± 0.08	2.5 ± 0.1	2.9 ± 0.1[c]	3.1 ± 0.2[c]	3.7 ± 0.2[c]	2.3 ± 0.08
18:0	2.2 ± 0.04	2.4 ± 0.05	2.4 ± 0.07	2.4 ± 0.1	2.3 ± 0.06	2.4 ± 0.06	2.3 ± 0.07
18:1	24.2 ± 0.5	22.6 ± 0.9	22.5 ± 1.0	22.1 ± 1.2	19.6 ± 1.1	16.6 ± 1.0[c]	20.5 ± 0.8
18:2n-6	13.4 ± 0.2[c]	12.0 ± 0.2	10.4 ± 0.4[c]	7.6 ± 0.2[c,d]	5.0 ± 0.2[c,e]	3.4 ± 0.2[c,f]	4.7 ± 0.2[g]
18:3n-3	0.1 ± 0.01	0.2 ± 0.01	0.2 ± 0.03	0.2 ± 0.01	0.3 ± 0.01	0.2 ± 0.06	0.2 ± 0.03
18:4n-3	0.9 ± 0.04[c]	0.6 ± 0.03	0.5 ± 0.03	0.5 ± 0.04	0.4 ± 0.02[c]	0.2 ± 0.06[c]	0.4 ± 0.02
20:2n-6	1.0 ± 0.04[c]	0.5 ± 0.02	0.4 ± 0.03[c]	0.2 ± 0.02[c,d]	0.1 ± 0.01[c]	0.0 ± 0.0[c]	0.1 ± 0.02[g]
20:3n-6	0.5 ± 0.07	0.5 ± 0.02	0.4 ± 0.02	0.3 ± 0.01	0.3 ± 0.01[c]	0.2 ± 0.05[c]	3.1 ± 0.8[g]
20:4n-6	0.6 ± 0.01[c]	0.4 ± 0.03	0.3 ± 0.06	0.3 ± 0.01	0.3 ± 0.01	0.2 ± 0.06[c]	1.4 ± 0.05[g]
20:5n-3	0.01 ± 0.01[c]	0.7 ± 0.02	1.5 ± 0.04[c]	2.5 ± 0.1[c,d]	3.6 ± 0.2[c,e]	5.6 ± 0.2[c,f]	1.6 ± 0.08
22:4n-6	0.3 ± 0.07[c]	0.1 ± 0.0	0.06 ± 0.03	0.2 ± 0.003	0.2 ± 0.01	0.3 ± 0.06	0.09 ± 0.02
22:5n-6	0.2 ± 0.05	0.1 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.4 ± 0.02[g]
22:5n-3	0.0 ± 0.0[c]	0.9 ± 0.02	1.5 ± 0.7[c]	2.1 ± 0.1[c,d]	2.7 ± 0.1[c,e]	3.8 ± 0.2[c,f]	1.5 ± 0.05
22:6n-3	0.06 ± 0.02[c]	0.5 ± 0.02	0.7 ± 0.02[c]	0.9 ± 0.04[c,d]	1.1 ± 0.05[c,e]	1.5 ± 0.060[c,f]	0.6 ± 0.03

[a]Values are expressed in % and represent group means ± SEM with n = number of dams.

[b]In this group the dietary 18:2n-6 was replaced partially by 18:3n-6.

Groups are significantly different by Tukey's t-test, $p < 0.05$: c, each group *vs* 0.25; d, 1.0 *vs* 0.5; e, 2.0 *vs* 1.0; f, 4.0 *vs* 2.0; g, 0.5 (18:3n-6) *vs* 0.5 (18:2n-6).

TABLE 3

Effects of Maternal Dietary n-3/n-6 Ratio on Selected Fatty Acid Composition of Brain Phosphatidylcholine in B6D2F$_2$ Mouse Pups on Day 32 Postconception[a]

	0.0 n = 9	0.25 n = 9	0.5 n = 6	1.0 n = 10	2.0 n = 6	4.0 n = 6	0.5[b] n = 9
14:0	2.3 ± 0.1[c]	3.1 ± 0.2	2.3 ± 0.2[c]	1.7 ± 0.1[c]	2.3 ± 0.2[c]	2.3 ± 0.1[c]	3.1 ± 0.3[g]
16:0	41.9 ± 0.3[c]	48.3 ± 0.4	41.1 ± 0.7[c]	38.0 ± 0.3[c,d]	40.7 ± 0.5[c,e]	41.3 ± 0.8[c]	47.1 ± 0.7[g]
16:1	4.6 ± 0.3	5.1 ± 0.2	3.8 ± 0.2[c]	3.8 ± 0.09[c]	4.4 ± 0.1	4.7 ± 0.1	5.4 ± 0.4[g]
18:0	10.5 ± 0.4[c]	7.2 ± 0.3	12.1 ± 0.4[c]	13.4 ± 0.2[c]	11.7 ± 0.2[c,e]	10.9 ± 0.3[c]	7.7 ± 0.6[g]
18:1	19.4 ± 0.3	20.2 ± 0.3	19.5 ± 0.2	20.1 ± 0.2	19.3 ± 1.8	21.5 ± 0.2	19.3 ± 0.4
18:2n-6	1.3 ± 0.05[c]	1.5 ± 0.03	1.3 ± 0.04	1.3 ± 0.03[c]	1.3 ± 0.03[c]	0.9 ± 0.1[c,f]	0.3 ± 0.05[g]
18:3n-3	0.1 ± 0.02	0.0 ± 0.0	0.3 ± 0.2[c]	0.1 ± 0.02[d]	0.1 ± 0.02	0.2 ± 0.04	0.0 ± 0.0[g]
18:4n-3	0.5 ± 0.03	0.5 ± 0.03	0.6 ± 0.02[c]	0.6 ± 0.02[c]	0.6 ± 0.02[c]	0.7 ± 0.02[c]	0.3 ± 0.07[g]
20:2n-6	0.4 ± 0.03	0.4 ± 0.04	0.5 ± 0.02	0.5 ± 0.01	0.1 ± 0.03[c,e]	0.3 ± 0.1[f]	0.0 ± 0.0[g]
20:3n-6	0.4 ± 0.02	0.5 ± 0.07	0.7 ± 0.01[c]	0.9 ± 0.01[c,d]	0.9 ± 0.06[c]	0.8 ± 0.03[c]	0.9 ± 0.05
20:4n-6	8.7 ± 0.2[c]	7.8 ± 0.2	6.5 ± 0.2[c]	6.0 ± 0.1[c]	4.7 ± 0.1[c,e]	3.8 ± 0.2[c]	9.0 ± 0.2[g]
20:5n-3	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.01[c]	0.2 ± 0.01[c,d]	0.4 ± 0.03[c,e]	0.04 ± 0.04[f]	0.7 ± 0.0[g]
22:4n-6	1.6 ± 0.07[c]	0.5 ± 0.02	0.9 ± 0.03[c]	0.7 ± 0.03[c]	0.3 ± 0.01[c,e]	0.2 ± 0.04[c]	0.0 ± 0.05
22:5n-6	2.5 ± 0.1[c]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.01[c]	0.8 ± 0.02[c,d]	1.0 ± 0.06[c]	1.1 ± 0.07[c]	0.1 ± 0.03[g]
22:6n-3	5.1 ± 0.2	5.1 ± 0.2	9.7 ± 0.7[c]	11.7 ± 0.3[c,d]	10.0 ± 0.3[c,e]	10.2 ± 0.6[c]	6.1 ± 0.3[g]

[a]Values are expressed in % and represent group means ± SEM with n = number of litters.

[b]In this group the dietary 18:2n-6 was replaced partially by 18:3n-6.

Groups are significantly different by Tukey's t-test, $p < 0.05$: c, each group *vs* 0.25; d, 1.0 *vs* 0.05; e, 2.0 *vs* 1.0; f, 4.0 *vs* 2.0; g, 0.5 (18:3n-6) *vs* 0.5 (18:2n-6).

TABLE 4

Effects of Maternal Dietary n-3/n-6 Ratio on Selected Fatty Acid Composition of Brain Phosphatidylethanolamine in B6D2F$_2$ Mouse Pups on Day 32 Postconception[a]

n-3/n-6 Ratio	0.0 n = 9	0.25 n = 9	0.5 n = 5	1.0 n = 10	2.0 n = 7	4.0 n = 7	0.5[b] n = 9
14:0	0.1 ± 0.05	0.7 ± 0.4	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.01	0.2 ± 0.1	0.1 ± 0.03
16:0	11.4 ± 0.3	10.5 ± 0.3	10.9 ± 0.4	11.6 ± 0.2	11.3 ± 0.4	11.2 ± 0.3	10.8 ± 0.1
16:1	0.4 ± 0.04	0.4 ± 0.1	0.5 ± 0.8	0.4 ± 0.03	0.4 ± 0.03	0.6 ± 0.6	0.4 ± 0.02
18:0	24.5 ± 0.5	23.7 ± 0.5	23.2 ± 0.9	25.2 ± 0.3	24.3 ± 0.6	22.0 ± 0.9	23.6 ± 0.9
18:1	8.6 ± 0.1	7.9 ± 0.3	9.2 ± 0.2[c]	9.5 ± 0.2[c]	9.5 ± 0.3[c]	10.0 ± 0.3[c]	7.9 ± 0.1[g]
18:2n-6	0.7 ± 0.03	0.6 ± 0.05	0.8 ± 0.03[c]	0.6 ± 0.03	0.6 ± 0.04	0.5 ± 0.04	0.2 ± 0.02[g]
18:3n-3	0.4 ± 0.8[c]	0.2 ± 0.03	0.4 ± 0.1	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.02	0.2 ± 0.01
18:4n-3	0.6 ± 0.1	0.4 ± 0.06	0.5 ± 0.02	0.6 ± 0.03	0.5 ± 0.03	0.6 ± 0.04	0.4 ± 0.02[g]
20:2n-6	0.6 ± 0.03[c]	0.3 ± 0.06	0.6 ± 0.05[c]	0.7 ± 0.04[c]	1.0 ± 0.07[c,e]	1.0 ± 0.04[c]	0.2 ± 0.01
20:3n-6	0.6 ± 0.02	0.5 ± 0.04	0.9 ± 0.02[c]	0.9 ± 0.03[c]	1.1 ± 0.06[c,e]	1.1 ± 0.03[c]	0.8 ± 0.03
20:4n-6	21.8 ± 0.1[c]	18.7 ± 0.7	18.0 ± 0.4	16.1 ± 0.3[c]	13.7 ± 0.2[c,e]	11.4 ± 0.6[c,f]	21.2 ± 0.4[g]
20:5n-3	0.0 ± 0.0	0.1 ± 0.03	0.3 ± 0.03	0.6 ± 0.02[c]	1.3 ± 0.07[c,e]	2.3 ± 0.2[c,f]	0.1 ± 0.01
22:4n-6	6.4 ± 0.07[c]	4.5 ± 0.1	3.3 ± 0.05[c]	2.4 ± 0.07[c,d]	1.4 ± 0.07[c,e]	0.9 ± 0.06[c,f]	5.1 ± 0.1[g]
22:5n-6	7.3 ± 0.2[c]	0.4 ± 0.06	0.2 ± 0.06	0.2 ± 0.02	0.1 ± 0.01	0.1 ± 0.02[c]	0.6 ± 0.01
22:5n-3	0.2 ± 0.04[c]	0.8 ± 0.06	1.4 ± 0.04[c]	2.0 ± 0.04[c,d]	3.0 ± 0.09[c,e]	3.8 ± 0.09[c,f]	0.8 ± 0.02[g]
22:6n-3	15.1 ± 0.5[c]	27.2 ± 0.7	28.1 ± 1.1	27.3 ± 0.5	30.1 ± 0.7	33.4 ± 0.8[c,f]	26.7 ± 0.9

[a]Values are expressed in % and represent group means ± SEM with n = number of litters.

[b]In this group the dietary 18:2n-6 was replaced partially by 18:3n-6.

Groups are significantly different by Tukey's t-test, $p < 0.05$: c, each group *vs* 0.25.; d, 1.0 *vs* 0.5; e, 2.0 *vs* 1.0; f, 4.0 *vs* 2.0; g, 0.5 (18:3n-6) *vs* 0.5 (18:2n-6).

dietary n-3/n-6 ratio increased. No consistent dose-response effects were observed on the levels of SFA in the brain. In PE and PC, levels of 18:1 increased with increasing levels of n-3; in the milk, levels of 18:1 decreased with increasing n-3, whereas those of 16:1 increased. Quantification of the individual phospholipid classes in the brain showed only a small range over the groups (wt%: PC: 30.5–34.8; PE: 26.1–28.2; PI: 2.3–2.8; PS: 7.7–8.6; SM: 3.1–4.9). Comparison of the two ratio 0.5 groups indicated that the effect of 18:3n-3 was to increase levels of 20:4n-6 in both milk and brain PC and PE. In PC, but not in PE, there was an accompanying slight decrease in levels 22:6n-3.

DISCUSSION

This study sought to examine the effects of varying maternal dietary long-chain n-3 to n-6 FA ratios on the incorporation of n-3 FA into the brains of the developing offspring. A question of particular interest was whether the brain would show the capacity to regulate its fatty acid composition at the higher dietary ratios. The results provide support for such regulation. While the brain showed continually increasing n-3/n-6 ratios with dietary increases, the maximum effect had occurred by 0.5 in PC and 0.25 in PE, with a significantly lower rate of increase beyond these values. Thus, as with the work done with 18:3n-3, the largest effect seen was in comparison with the deficient group. This is supported by the observation that 22:5n-6 was only present to any appreciable extent in the ratio 0 group.

Although 20:5n-3 was the major n-3 FA in the milk, 22:6n-3 predominated in the brain; levels of 20:5n-3 in the brain were generally low, exceeding 1% in PE only at the higher ratios. The companion study on the livers of these animals demonstrated that 22:6n-3 was also the major n-3 FA in the liver, with the maximum 22:6n-3 content seen at ratio 0.5 (26). Although only trace amounts of 20:5n-3 and 22:6n-3 were found in the milk of ratio 0, the brains of these pups showed 5.1% and 15.1% 22:6n-3 in PC and PE, respectively. These data show that there is considerable accretion of long-chain n-3 PUFA during prenatal brain growth, and that dietary n-3 deficiency during gestation may be offset partially by selective retention of 22:6n-3 by the dam, possibly in the liver (30). The absence of n-3 postnatally in the milk of these dams may be explained by depletion of these stores, or, alternatively, by mechanisms which vary in terms of promoting the availability of stored PUFA prenatally to the fetus as compared with postnatally to the milk. Increases in n-3 were generally accompanied by decreases in n-6, particularly of 20:4n-6, thereby reflecting the similar n-3/n-6 reciprocity in the diet. It was shown recently in rats that with a minimal level of 18:2n-6 (0.3% of calories) the amount of 20:4n-6 in the brain remained constant, and, beyond a minimum, was independent of levels of dietary 18:3n-3 (31). This suggests that the present effects on 20:4n-6 may be due specifically to long-chain n-3. Interestingly, levels of 20:4n-6 were increased in both milk and brain by partial replacement of dietary 18:2n-6 with 18:3n-6. There are two possible mechanisms which may account for this, both related to desaturase activity. It is known that 20:5n-3 and 22:6n-3 inhibit Δ6 and Δ5 desaturases (32,33), which reduces the formation of 20:4n-6 from 18:2n-6; the presence of large amounts of 18:2n-6 may result in substrate inhibition of the Δ6 desaturase (34). In both instances, the provision of 18:3n-6 would avoid the effects of a decline in Δ6 desaturase activity. In contrast with the other n-6, 20:3n-6 increased. This replicates our previous findings and suggests inhibition of Δ5 desaturase activity by FA derived from fish oil (23,25). The general absence of effects on the SFA is consistent with other work (35).

With respect to the increase in 18:1 seen with increasing n-3, a similar finding has been reported for chick brain (36).

The data on the milk lipids support previous work in showing an effect of maternal dietary PUFA composition (37,38); they also extend these findings by demonstrating the dose-response nature of this relationship. In contrast with the tendency of the n-3/n-6 ratio to level off in the brain, that of the milk lipids showed a strong linear increase with increasing dietary ratios. These results suggest therefore that it is the metabolism of the pup, not the dam, which is regulating either supply or incorporation into the developing brain.

No systematic effects on overall growth and development were observed, except at ratio 1.0, as seen by the smaller litter size. Also, relative to body weight, increasing the n-3/n-6 ratio resulted in a very small decrease in brain weight. We had shown previously that n-3 supplementation slightly accelerated eye-opening, and that this correlated positively with other indices of sensorymotor development (22). However, in the present study, no effect on the rate of eye-opening was observed.

The diets used in this study were clearly experimental in that they provided only long-chain n-3 FA, with no 18:3n-3, and the n-3/n-6 ratios beyond 0.25 were high. Moreover, species differences in desaturase activities (39) may limit the generalizability of these results. Nevertheless, the findings may be of relevance to questions concerning the provision of long-chain n-3 FA in human infant feeding. They suggest that provision of n-6 FA partially as 18:3n-6 may help to offset the decrease in 20:4n-6 observed with dietary long-chain n-3 FA. They also clearly support a trend toward regulatory limits on the incorporation of long-chain n-3 FA into the mouse brain, showing a maximum response to maternal dietary n-3/n-6 ratios of 0.25–0.5

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Effect of Maternal Dietary Fats with Variable n-3/n-6 Ratios on Tissue Fatty Acid Composition in Suckling Mice

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This report examines the distribution of n-3 and n-6 fatty acids in heart, kidney and liver phosphatidylcholine and phosphatidylethanolamine of suckling mice from dams fed a fat-supplemented diet with variable n-3/n-6 ratios. After conception and throughout the pregnancy and lactation period, dams were fed a fat-free liquid diet supplemented with 20% by energy of oil mixtures (fish oil concentrate, rich in 20:5n-3 and 22:6n-3, and safflower oil concentrate, rich in 18:2n-6). The diets contained similar amounts of combined n-3 and n-6 fatty acids but variable ratios of n-3 to n-6 fatty acids (0, 0.25, 0.5, 1, 2 and 4). In 12-day-old suckling mice, as the n-3/n-6 ratio in the maternal diet increased (up to approx. 0.5), the tissue levels of 20:5n-3, 22:5n-3 and 22:6n-3 increased, whereas those of 18:2n-6 and 20:4n-6 decreased. The responses were similar in both phospholipid subclasses, but varied between different tissues. Generally, the n-3/n-6 ratios were significantly greater in pup tissues than in milk fat, indicating preferential incorporation of n-3 over n-6 fatty acids into phospholipids during growth. However, the incorporation of n-3 fatty acids in pups was significantly suppressed whereas that of n-6 fatty acids was increased when 18:2n-6 was replaced by its Δ6-desaturation product, 18:3n-6 (concentrated from evening primrose oil), as the source of n-6 fatty acid. This result suggests that Δ6 desaturase activity in neonate tissues is low, and consequently, the metabolism of 18:2n-6 to longer chain n-6 fatty acids is reduced. The preformed long-chain n-3 fatty acids, which bypass Δ6-desaturation, were thus, preferentially incorporated into tissue phospholipids.
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Two essential fatty acids, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), represent the parent acids of n-6 and n-3 metabolites, respectively. It is generally agreed that both n-6 and n-3 fatty acids are metabolized by the same enzyme systems (1,2). Previously, the interaction between n-6 and n-3 fatty acids has been studied mostly on adult and post-weaned growing animals; few have been carried out on pre-weaning neonates. Since long-chain n-6 and n-3 fatty acids constitute the major structural components in neural tissues (3–5), and the active brain growth (spurt) in mice occurs immediately after birth, changes in fatty acid metabolism would have a profound effect on postnatal development.

Nutrients are supplied to the preweaning neonate solely from its mother's milk, and therefore, the quality and quantity of the milk fat dictate the fatty acid metabolism in neonates. In normal human and animal milk, the levels of n-3 fatty acids in comparison with n-6 fatty acids are very low. This may be a result of the abundant presence of n-6 fatty acids in the maternal diet which suppresses the metabolism and incorporation of n-3 fatty acids into milk fat and consequently limits the supply of n-3 fatty acids to the neonates through the milk. However, the long-chain n-3 fatty acid content of milk can be enriched through dietary supplementation of preformed long-chain n-3 fatty acids, such as eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), to the maternal diet (6). We have shown previously that increasing maternal n-3 fatty acid intake accelerates the behavioral development in neonates (7).

Increasing evidence has indicated that excessive and prolonged intake of long-chain n-3 fatty acids may produce some adverse effects in humans and animals (8). Since maternal dietary fat modulates the milk n-3 and n-6 fatty acid content, and the preweaned neonate obtains nutrients solely from its mother's milk, varying the fatty acid composition of the maternal diet would affect fatty acid metabolism in the preweaned neonate. In the preceding paper (9), we describe the changes of pup brain fatty acids in response to maternal diets supplemented with a constant level of combined n-3 and n-6 fatty acids but with variable n-3/n-6 ratios (0, 0.25, 0.5, 1, 2 and 4). In this communication, we report the changes of n-3 and n-6 fatty acids in heart, kidney and liver phospholipid subclasses of the same suckling mice (9). The n-3/n-6 ratios of 0.25 and 4 used are approximately 1- and 16-fold, respectively, of that reported in a previously published study (7).

MATERIALS AND METHODS

Male and female mice (B6D2 F_1 hybrid, 28–32 wk old) were purchased from Charles River Breeding Laboratories (St. Constant, Quebec, Canada). Mice were group-housed in a constant temperature (22°C) room with a reversed 12-hr light/12-hr dark cycle, and had free access to water and lab chow (Purina No. 5001). Animals were mated and conception was confirmed by the presence of a vaginal plug (day 0 post-conception). The pregnant dams were then housed individually in opaque plastic cages (29 × 18 × 13 cm) containing Beta-Chip hardwood bedding and toilet tissue for nesting material. The dams were randomly assigned to different experimental groups (number of animals in each group varied from 6 to 10), and fed a liquid diet (0.65 kcal/g body wt per day), supplemented with 20% by calories of 6 different oil mixtures. The composition of the liquid diet (F 2187) formulated by BioServ (Frenchtown, NJ) has been described previously (10). The diet contained (by energy) 20% fat-free casein, 20% fat, and 60% maltose-dextrin including mineral and vitamin mixtures. In addition, 4 IU/g oil of α-tocopherol (or approximately 96 IU/L diet) were also added to the diet (contains 56 IU/L), as antioxidant. The fat was prepared by mixing variable amounts of olive (containing 13% 18:2n-6), safflower (containing 69.7% 18:2n-6), and concentrated fish oils (containing 41.2% 20:5n-3 and 6.1% 22:6n-3, provided by Callanish Ltd., Bresclete, Isle of Lewis, Scotland), to provide constant combined n-3 and n-6 levels (approximately 40% of total fatty acids) but with variable n-3/n-6 ratios (0, 0.25, 0.5, 1, 2, and 4). In all diets, at least 1.5% (en) of n-6 fatty acids was provided to ensure that the

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Abbreviations: MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

animals were free of essential fatty acid deficiency. The detailed fatty acid compositions of the oil mixtures are described in the preceding paper (9). The diets were freshly prepared every other day and stored under nitrogen at 5°C. In a separate group, dams (n = 6) were fed a diet with a n-3/n-6 ratio of 0.5 and a 18:3n-6 concentrate (containing 68.7% 18:3n-6 and 19% 18:2n-6, derived from evening primrose oil and prepared by Callanish Ltd.) as a source of n-6 fatty acid. The purpose of this manipulation was to examine whether the incorporation of n-3 and n-6 fatty acids might be affected by the ability of tissues to metabolize (Δ6-desaturate) 18:2n-6.

Birth occurred on day 19 or 20 (post-conception), and litters were culled to six (3 males and 3 females). From day 20 until day 32, the lactating dams were fed the appropriate diet at 0.975 Kcal/g of body weight/day. On day 32, two pups (one male and one female) in each litter were selected randomly, anesthetized with Halothane and then decapitated. The brain, liver, kidney and heart were excised, pooled and frozen at −50°C until lipid analysis. The brain samples and the remaining pups (n = 4 in each litter) were subjects of the behavioral study as described in the preceding paper (9). On day 33, all dams were milked under light anesthesia after receiving 0.4 IU/kg body weight of oxytocin following the procedure described previously (11).

Total lipids of all tissues and milk were extracted using a method described by Folch *et al.* (12). The liver, kidney and heart phospholipid subclasses, phosphatidylcholine (PC), phosphatidylethanolamine (PE) phosphatidylinositol and phosphatidylserine, were separated by thin-layer chromatography using the solvent system, chloroform/ethanol/water/triethylamine (4:5:1:4, by vol) (13). The lipid fractions were identified by comparison with authentic standards after visualization with 2′,7′-dichlorofluorescein (0.025% in ethanol). Milk fat and two major phospholipid subclasses (PC and PE) were transmethylated as described by Morrison and Smith (14). The fatty acid methyl esters were analyzed by gas-liquid chromatography using a Hewlett-Packard gas chromatograph (model 5890, Avondale, PA) equipped with a flame-ionization detector and a fused silica capillary column (0.25 mm i.d. × 15 m) coated with SP-2230 (Supelco Canada, Oakville, Ontario) as described previously (15). Fatty acids were identified by comparison of retention times with those of authentic standards (Nu-Chek Prep, Elysian, MN).

The concentration of total phospholipids, and the percent distribution of phospholipid subclasses were measured by high-performance liquid chromatography (System Gold, Beckman Canada, Mississauga, Ontario) using a Si Ultrasphere column (5 microns, 4.6 mm i.d. × 250 mm, Beckman) and a mass detector (ACS 750/14, Applied Chromatography Systems Ltd, Lutons, Bds, United Kingdom) as described previously (16).

One way analysis of variance was used to examine the relationship between the dietary n-3/n-6 ratios and fatty acid levels in tissue PC and PE. The effects of 18:2n-6

TABLE 1

Concentration (mg/g tissue) and Distribution (% total) of Major Phospholipid Subclasses in Pup Liver, Kidney and Heart[a]

	Dietary n-3/n-6 ratio					
	0	0.25	0.5	1	2	4
n	9	9	6	10	7	7
	Tissue phospholipid concentration (mg/g)					
Liver	17.9[b]	17.1	19.4	16.9	18.6	17.1
Kidney	7.7	6.3	6.4	7.9	6.8	6.9
Heart	7.6	8.0	7.7	8.1	7.9	8.4
	Distribution (% total phospholipids)					
Liver						
PC	52.6	50.3	49.6	50.4	49.8	48.9
PE	29.4	31.1	31.0	30.3	30.7	30.8
PI	4.8	5.1	5.0	5.0	4.9	5.1
PS	0.9	1.0	2.0	1.0	1.0	1.3
SM	1.9	1.7	2.4	2.5	2.0	2.1
Others	10.4	10.8	11.0	10.8	11.6	11.8
Kidney						
PC	38.6	40.2	39.4	38.8	39.0	39.1
PE	30.8	33.3	32.8	30.7	32.1	33.7
PI	6.0	4.4	4.8	5.3	4.7	5.6
PS	5.2	4.0	5.0	4.7	3.8	5.3
SM	8.8	5.3	4.7	8.3	7.9	4.4
Others	10.6	12.8	13.3	12.2	12.5	11.9
Heart						
PC	50.8	52.6	53.2	54.1	49.6	52.8
PE	38.7	37.2	38.1	38.1	39.7	37.4
PI	3.8	4.2	3.7	4.4	4.2	4.2
Others	6.7	6.0	5.0	6.4	6.7	5.6

[a]Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; others, include cardiolipins and other phosphorus-containing lipids.

[b]Each value represents the mean of n determinations, where n represents the number of litters and each litter represents a pooled sample from one male and one female pup.

and 18:3n-6 in maternal diet on n-3 and n-6 fatty acid distribution in pup tissue phospholipids were assessed using the Student's t-test.

RESULTS

Table 1 shows the tissue phospholipid concentrations and distributions of phospholipid subclasses in 12-day-old (32-day postconception) suckling mice from dams fed a fat-supplemented liquid diet with variable n-3/n-6 ratios. Overall, no consistent differences were observed between various dietary groups. Body weight, liver, heart and kidney weights were also not significantly different between pups from dams fed different dietary fats (data not shown).

The changes in the major n-3 and n-6 fatty acids in liver PC and PE in response to changes in the n-3/n-6 ratio in the maternal diet are shown in Figure 1. Increasing the dietary n-3/n-6 ratio increased the levels of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and total n-3 fatty acids, but decreased those of polyunsaturated fatty acids (PUFA) and n-6 fatty acids. n-6 Fatty acids (18:2n-6 and 20:4n-6) decreased more rapidly when the dietary n-3/n-6 ratio increased from 0 to 0.5 than from 0.5 to 4. The major n-3 fatty acid in liver PC and PE was 22:6n-3, followed by 20:5n-3 and 22:5n-3. The levels of 22:6n-3 rose and reached a maximum very rapidly when only a small amount of long chain n-3 fatty acids was included in the diet (*i.e.*, when the dietary n-3/n-6 ratio increased from 0 to 0.5). When the dietary n-3/n-6 ratio increased from 2 to 4, 22:6n-3 levels remained maximal in PE, whereas they fell in PC. The levels of 20:5n-3 and 22:5n-3 increased very rapidly when the dietary n-3/n-6 ratio increased from 0 to 1, and leveled off when the n-3/n-6 ratio increased from 1 to 4.

In kidney (Fig. 2) and heart (Fig. 3) phospholipids, increasing the dietary n-3/n-6 ratio increased the levels of total n-3 fatty acids and decreased those of n-6 fatty acids, but did not significantly affect the levels of SFA, MUFA and PUFA. In general, the pattern of change of major n-3 and n-6 fatty acids in kidney (Fig. 2) and heart (Fig. 3) was similar to that in liver phospholipids (Fig. 1). Both 18:2n-6 and 20:4n-6 levels fell rapidly whereas 20:5n-3, 22:5n-3 and 22:6n-3 rose rapidly as the dietary n-3/n-6 ratio increased. The extent of change was considerably smaller for 18:2n-6 than for 20:4n-6. Among the n-3 fatty acids, 20:5n-3 in kidney PC reached the same level as 22:6n-3, when the n-3/n-6 ratio in the maternal diet exceeded 1. In kidney PE, the level of 20:5n-3 continued to rise and exceeded that of 22:6n-3. The major n-3 fatty acid in heart

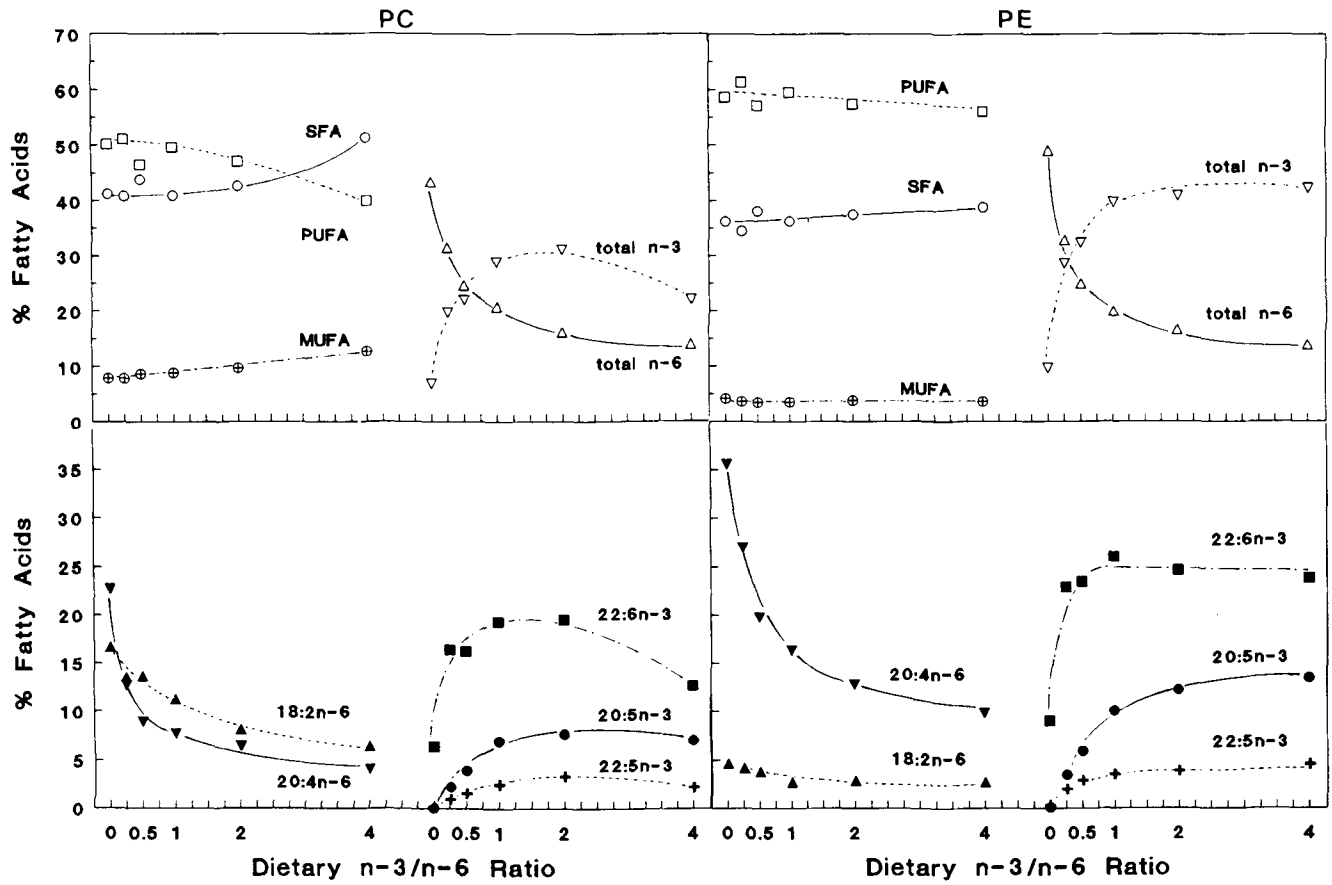

FIG. 1. The distribution (% of total fatty acids) of saturated (SFA, ○), monounsaturated (MUFA,⊕), polyunsaturated (PUFA, □), total n-3 (▽) and n-6 (△) fatty acids, and the major n-6 (18:2n-6, ▲; and 20:4n-6, ▼) and n-3 (20:5n-3, ●; 22:5n-3, +; and 22:6n-3, ■) fatty acids in liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of suckling mice from dams fed a liquid diet supplemented with 20% (energy) fats, but with variable n-3/n-6 ratios. Each data point represents the mean of 9, 9, 6, 10, 6, or 6 pooled samples of one male and one female pup from dams fed a diet with n-3/n-6 ratio at 0, 0.25, 0.5, 1, 2 or 4, respectively.

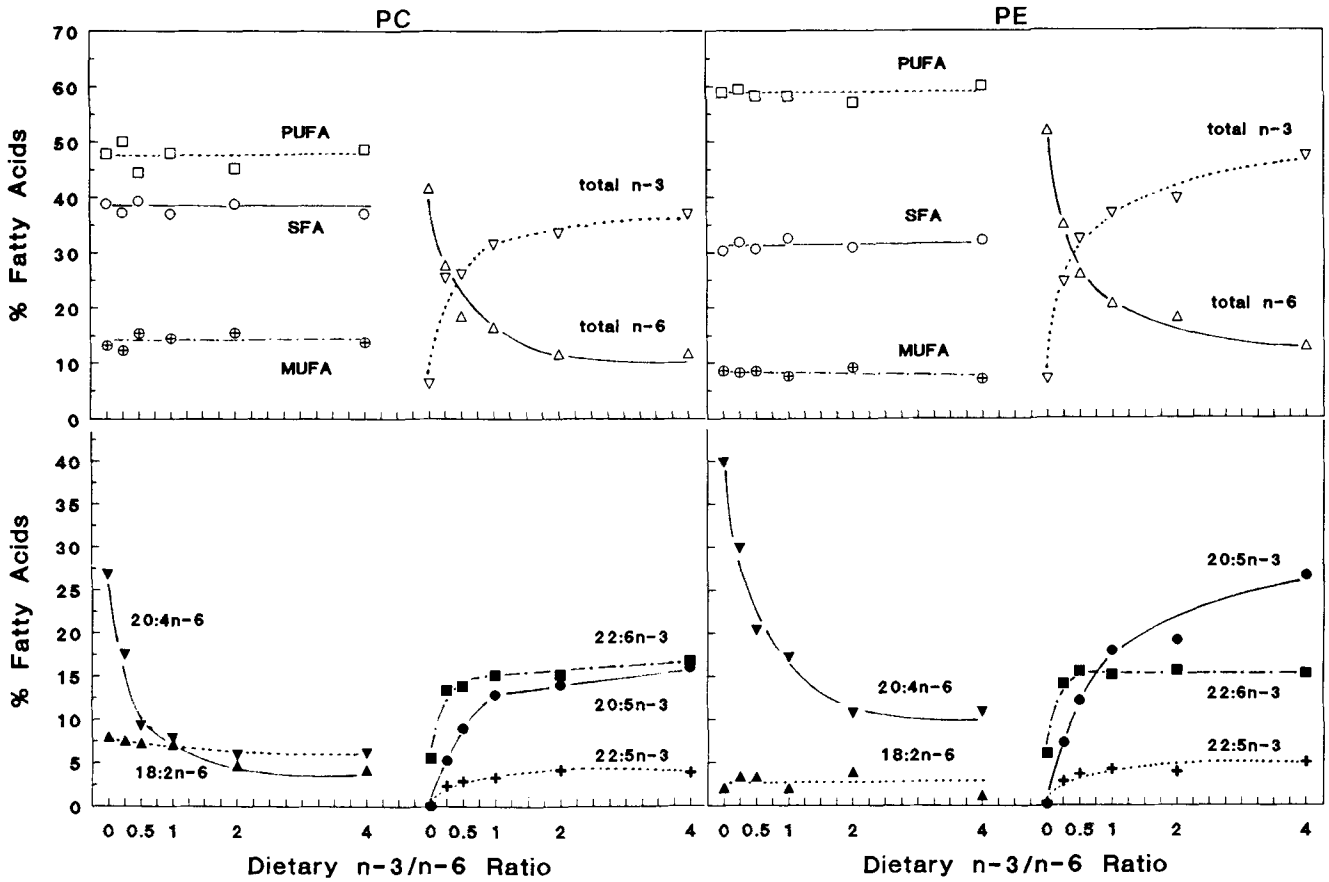

FIG. 2. The distribution (% of total fatty acids) of saturated (SFA, ○, monounsaturated (MUFA, ⊕), polyunsaturated (PUFA, □), total n-3 (▽) and n-6 (△) fatty acids, and the major n-6 (18:2n-6, ▲; and 20:4n-6, ▼) and n-3 (20:5n-3, ●; 22:5n-3, +; and 22:6n-3, ■) fatty acids in kidney phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of suckling mice from dams fed a liquid diet supplemented with 20% (energy) fats, but with variable n-3/n-6 ratios. Each data point represents the mean of 9, 9, 6, 10, 6, 6 pooled samples of one male and one female pup from dams fed a diet with n-3/n-6 ratio at 0, 0.25, 0.5, 1, 2 or 4, respectively.

phospholipids was 22:6n-3, followed by 22:5n-3. Both 22:5n-3 and 22:6n-3 rose rapidly to maximal levels when only a small amount of long-chain n-3 fatty acids was included in the diet (n-3/n-6 ratio ≥ 0.25). The levels of 20:5n-3 in heart phospholipids were low in comparison with the other two n-3 acids, and were less responsive to changes of n-3/n-6 ratios in the maternal diet.

Figure 4 illustrates the response of tissues in suckling mice to maternal dietary change, plotting the n-3/n-6 ratios in tissue PC and PE of suckling mice against the n-3/n-6 ratios in the maternal diet. These relationships were then compared with those of milk fat. Results indicate that there existed a linear relationship between the ratio of n-3/n-6 fatty acids in milk fat and that in the maternal diet. However, the increases of the n-3/n-6 ratios in response to the maternal dietary change were significantly greater than those of milk fat in tissue PC (except in liver PC, when the n-3/n-6 ratio in the maternal diet exceeded 4) and PE, and greater in the heart than in other tissue phospholipids.

Figure 5 shows the effects of maternal dietary supplementation of either 18:2n-6 or 18:3n-6 as the main source of n-6 fatty acids (n-3/n-6 ratio = 0.5) on the distributions of major n-6 and n-3 fatty acids in pup liver, kidney and heart PC and PE. In general, the levels of 20:3n-6 and 20:4n-6 were consistently greater whereas those of long-chain n-3 fatty acids (20:5n-3, 22:5n-3 and 22:6n-3) were consistently lower in pup tissue phospholipids from the dams fed an 18:3n-6 enriched diet than from those fed an 18:2n-6 enriched diet. These differences are again illustrated in Figure 6, where the sum of tissue n-6 and n-3 fatty acids and the n-3/n-6 ratios in these animals are compared.

DISCUSSION

Figures 1-3 show that the levels of 20:4n-6 in neonatal tissue phospholipids were significantly reduced when the n-3/n-6 ratio in the maternal diet was increased from 0 to 0.5. Pup tissue 20:4n-6/18:2n-6 ratios in all phospholipids fell in a similar fashion as maternal dietary n-3/n-6 ratio increased. The reduction of 20:4n-6 in tissue phospholipids may reflect a reduced conversion from 18:2n-6. Increasing dietary intake of 20:5n-3 and 22:6n-3 has been shown to inhibit the activity of Δ6 and Δ5 desaturases (17,18). However, when the dietary n-3/n-6 ratio increased 8-fold from 0.5 to 4, 20:4n-6 further decreased only to a limited extent in tissue phospholipids. This finding in conjunction

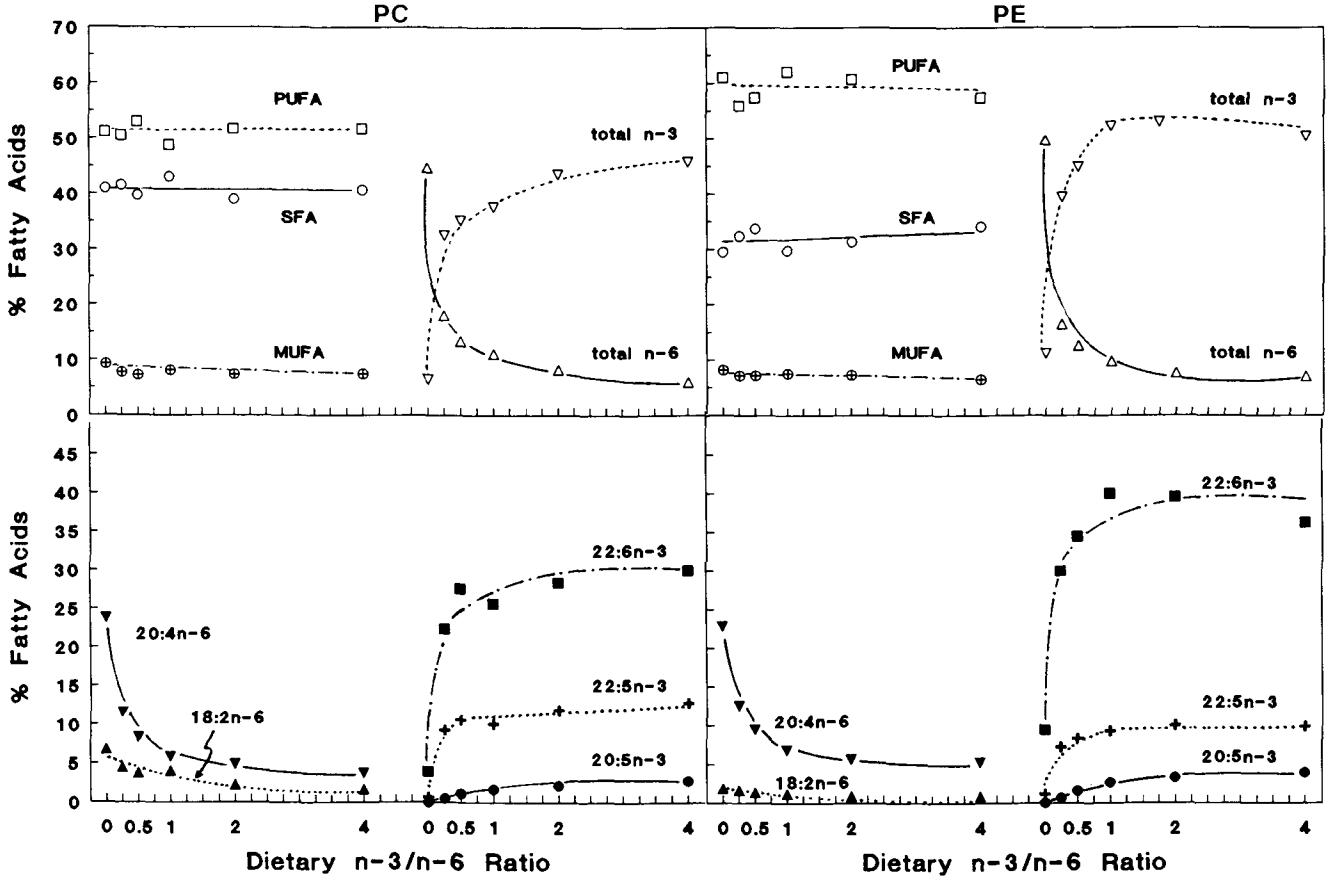

FIG. 3. The distribution (% of total fatty acids) of saturated (SFA, ○), monounsaturated (MUFA, ⊕), polyunsaturated (PUFA, □), total n-3 (▽) and n-6 (△) fatty acids, and the major n-6 (18:2n-6, ▲; and 20:4n-6, ▼) and n-3 (20:5n-3, ●; 22:5n-3, +; and 22:6n-3, ■) fatty acids in heart phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of suckling mice from dams fed a liquid diet supplemented with 20% (energy) fats, but with variable n-3/n-6 ratios. Each data point represents the mean of 9, 9, 6, 10, 6, 6 pooled samples of one male and one female pups from dams fed a diet with n-3/n-6 ratio at 0, 0.25, 0.5, 1, 2 or 4, respectively.

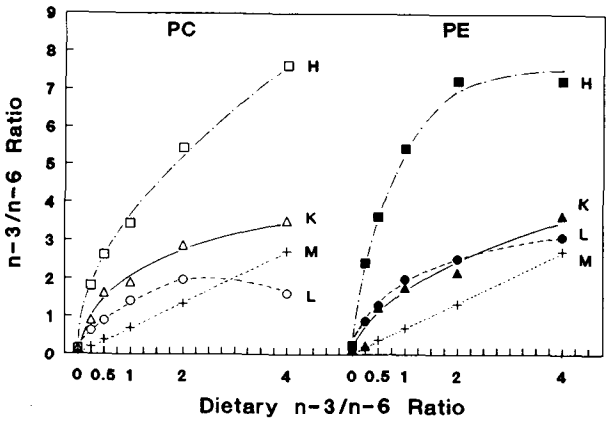

FIG. 4. The n-3/n-6 ratios in liver (L, ○, ●), kidney (K, △, ▲), and heart (H, □, ■) phosphatidylcholine (open symbols) and phosphatidylethanolamine (solid symbols) of suckling mice from dams fed a liquid diet supplemented with 20% (energy) fats, but with variable n-3/n-6 ratios (0, 0.25, 0.5, 1, 2 and 4). The n-3/n-6 ratios in milk fat (M, +) in response to n-3/n-6 changes in maternal diet are also shown.

with an insignificant change of the ratio of 20:4n-6/18:2n-6 in tissue phospholipids suggests that there was no further inhibition on the conversion of 18:2n-6 to 20:4n-6 in these dietary groups. The reduction of 20:4n-6 in tissue phospholipids was due to the fact that 20:5n-3 and 22:6n-3 competed with 20:4n-6 for incorporation into phospholipids. The competition between n-3 fatty acids and 20:4n-6 presumably outweighed the preferential effect of the phospholipid synthesis enzyme, acyltransferase, which favors the 20:4n-6 as substrate (19).

Figure 4 shows a direct relationship between milk fat and the n-3/n-6 ratio of the maternal diet. This relationship is in agreement with the report that milk fatty acid composition is regulated by the dietary n-3/n-6 ratio (6). However, at any given dietary n-3/n-6 ratio, the milk fat n-3/n-6 ratio is consistently lower than that of the maternal diet, suggesting that the dams preferentially incorporate n-6 fatty acids into milk fat over n-3 fatty acids. Despite this, the n-3/n-6 ratio in pup tissue phospholipids was consistently greater than that in milk fat. This suggests that milk long-chain n-3 fatty acids were more efficiently incorporated into and retained in

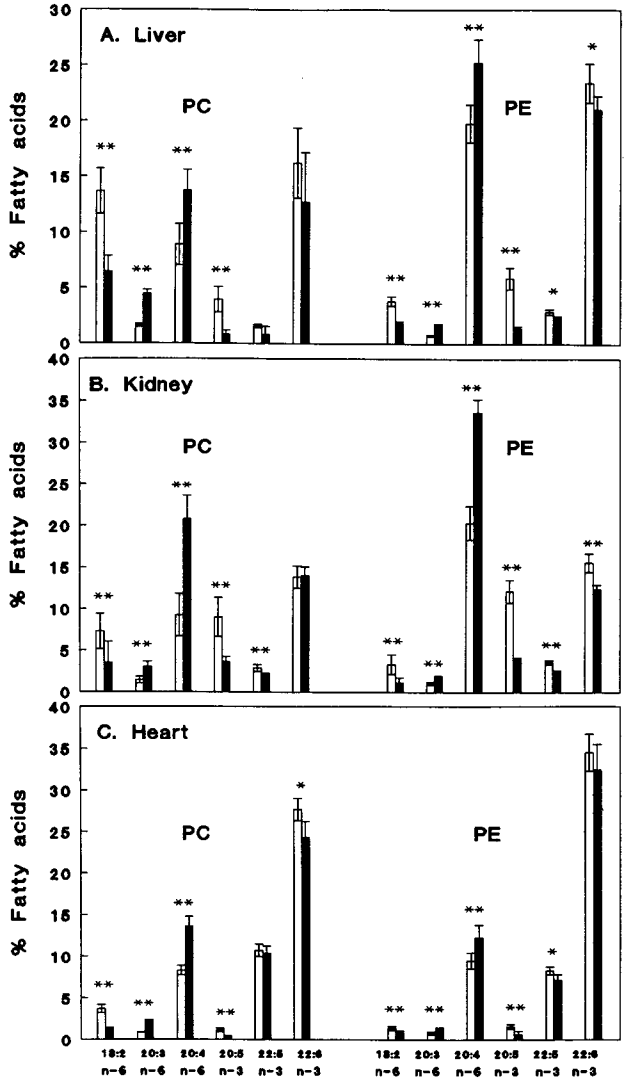

FIG. 5. The distribution (% of total fatty acids) of major n-6 (18:2n-6, 20:3n-6 and 20:4n-6) and n-3 (20:5n-3, 22:5n-3, and 22:6n-3) fatty acids in liver (panel A), kidney (panel B) and heart (panel C) phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of suckling mice from dams fed a liquid diet supplemented with 20% (energy) fat (n-3/n-6 ratio = 0.5). The source of n-3 fatty acids was from the concentrated fish oil while that of n-6 fatty acids was from either 18:2n-6 rich oil (□) or 18:3n-6 rich oil (■). Values represent means ± SD of 6 determinations. *, ** significantly different at $p<0.05$ or 0.01 respectively.

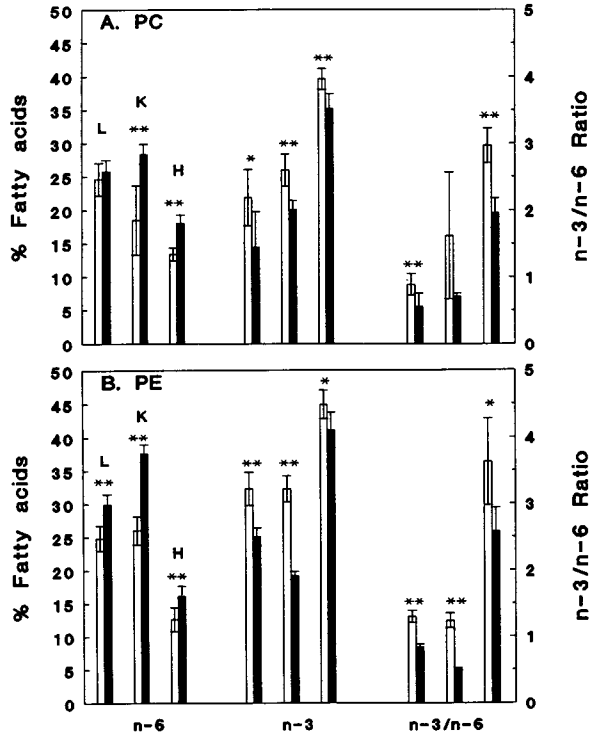

FIG. 6. The sum of n-6 and n-3 fatty acids and n-3/n-6 ratios in liver (L), kidney (K), and heart (H) phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of suckling mice from dams fed a liquid diet supplemented with 20% (energy) fats (n-3/n-6 ratio = 0.5). The source of n-3 fatty acids was from the concentrated fish oil while that of n-6 fatty acids was from either 18:2n-6 rich oil (□) or 18:3n-6 rich oil (■). Values represent means ± SD of 6 determinations. *, ** significantly different at $p<0.05$ or 0.01 respectively.

membrane phospholipids in the suckling mice than were milk n-6 fatty acids.

Figure 4 also shows the rate of increase of the n-3/n-6 ratio in different tissues in response to increasing the n-3/n-6 ratio in the maternal diet. The increased incorporation of n-3 fatty acids was compensated by a reduction in 20:4n-6 (and to a lesser extent, 18:2n-6). The change in heart phospholipids was the most striking among the three tissues examined (Fig. 3). The incorporation of n-3 fatty acids into heart tissues continued to rise at dietary n-3/n-6 ratios over 1.0. In kidney phospholipids, the n-3/n-6 ratios increased in parallel to but higher than those in milk fats. In liver phospholipids, the n-3/n-6 ratios leveled off when the n-3/n-6 ratios in the maternal diet exceeded 2. The difference in incorporation of n-3 and n-6 fatty acids might be regulated in part by the ability of tissues to metabolize 18:2n-6 and 18:3n-3. Heart tissue has only a limited ability for fatty acid desaturation (20); 20:4n-6 must be derived from plasma lipids after desaturation-elongation of 18:2n-6 in liver. When the supply of 20:4n-6 was reduced in milk from dams fed a diet with a high n-3/n-6 ratio, the high rate of acylation of 22:6n-3 and other n-3 fatty acids into heart phospholipids probably resulted from the high affinity of heart sarcoplasmic reticulum for n-3 PUFA (21–26). In kidney PE, the levels of 20:5n-3 continued to increase while 22:6n-3 reached and stayed at the maximal level (Fig. 2); this may be due to a portion of the 22:6n-3 being retroconverted into 20:5n-3 (27).

Previously, Sinclair (28) had shown that the uptake of labelled 22:6n-3 into brain lipids was greater in 16–17 day-old suckling rats than in adult rats. The accretion of n-3 fatty acids may depend upon the stage of growth as well as the activity of desaturation which tends to decrease as growth progresses (29). The view that low Δ6-desaturase activity may be responsible for higher incorporation of long-chain n-3 fatty acids is supported by comparing the

effect of dietary supplementation with 18:2n-6 and its Δ6 desaturation product, 18:3n-6 (Fig. 5 and 6). The incorporation of n-3 was significantly higher when the dietary source of n-6 fatty acids was 18:2n-6 in comparison with 18:3n-6. Since n-3 fatty acids (20:5n-3 and 22:6n-3) supplemented in the diet had already undergone Δ6 desaturation whereas the n-6 fatty acids (18:2n-6) had not, a decreased formation of 20:4n-6 reduces the ability of n-6 fatty acids to compete with preformed long-chain n-3 fatty acid for incorporation into tissue phospholipids (19).

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Esterification of Fatty Acids by Bovine Intramuscular and Subcutaneous Adipose Tissues[1]

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Exogenous fatty acid esterification in intramuscular and subcutaneous adipose tissues from 72-hr fasted or *ad libitum* fed Angus cattle was investigated. Intramuscular (interfascicular) and subcutaneous adipose tissue snips were obtained from the longissimus dorsi muscle and were incubated with radioisotopically labeled fatty acids (palmitate, stearate, oleate, linoleate or linolenate) at three different concentrations (0.3 mM, 0.6 mM and 2.0 mM) to assess rates of fatty acid incorporation into glycerolipids. Rates of fatty acid esterification *in vitro* increased with fatty acid concentration in both intramuscular and subcutaneous adipose tissues. For all of the fatty acids investigated, triglycerides were the predominant products (60–85%). Subcutaneous adipose tissue had larger adipocytes and more actively ($P<0.05$) esterified fatty acids, with the exception of palmitate, than intramuscular adipose tissue. The rate of palmitate esterification was not different between tissues, although intramuscular adipose tissue esterified a greater proportion ($P<0.10$) of palmitate as triglyceride (85%) than did subcutaneous adipose tissue (75%). Relative rates of incorporation of fatty acids into lipids in intramuscular and subcutaneous adipose tissues were: palmitate > linolenate > linoleate > stearate. In general, 72-hr fasting did not significantly reduce the rates of fatty acid incorporation in bovine adipose tissues. Results of this study revealed that: i) rates of exogenous fatty acid incorporation into adipose tissue lipids were dependent on the medium fatty acid concentration and adipose tissue depot; and ii) the relative esterification rates of the various fatty acids *in vitro* did not necessarily reflect the proportion of these fatty acids in bovine adipose tissues.
***Lipids* 27, 111–116 (1992).**

Evidence has accumulated to indicate that intramuscular (interfascicular) adipocytes represent a cell population different from the more extensively investigated subcutaneous adipocytes. Subcutaneous adipose tissue synthesizes fatty acids *de novo* at four to ten times the rate observed for intramuscular adipose tissue (1–3), even at a constant cell volume (3). Additionally, glucose is utilized more readily for fatty acid biosynthesis in bovine intramuscular adipose tissue than in subcutaneous adipose tissue (1,3). Glucose, the primary source of glycerol 3-phosphate for glycerolipid biosynthesis, has been shown to stimulate lipogenesis in bovine subcutaneous adipose tissue (3–6). Furthermore, fatty acids of dietary origin are readily accumulated into the perirenal and omental adipose tissues from ruminants (7,8). It is possible that the dissimilarity in preference for lipogenic substrates between intramuscular and subcutaneous adipose tissues influences the rate of esterification of exogenous fatty acids into these tissues.

Fasting has been shown to cause a marked reduction in the rate of fatty acid esterification in adipose tissue from swine (9) and rats (10) *in vitro*. Up to 55% and 48% suppression of the esterification of palmitate and glyceride-glycerol synthesis, respectively, were observed in subcutaneous adipocytes from pigs fasted for 48 hr (9). In ruminants, studies on fasting focused primarily on its effect on fatty acid synthesis (11–13) and oxidation (14,15). There is no information concerning fatty acid uptake and incorporation into bovine adipose tissue *in vitro*.

This study was conducted to quantify rates of exogenous fatty acid esterification in bovine intramuscular and subcutaneous adipose tissues *in vitro*. The effect of short term fasting on the glycerolipid synthesis of these two adipose tissues was also investigated to document the feasibility of a fasting-refeeding scheme in modifying the fatty acid composition of bovine adipose tissues.

MATERIALS AND METHODS

Chemicals. Bovine insulin (25.8 USP IU/mg) and bovine serum albumin (fraction V; fatty acid-, nuclease- and protease-free) were purchased from Calbiochem (La Jolla, CA). Radioisotopically-labeled compounds, [9,10(n)-^{3}H]-palmitate, [9,10(n)-^{3}H]oleate and [1-^{14}C]linolenate, were supplied by New England Nuclear (Boston, MA); [1-^{14}C]-linoleate and [1-^{14}C]stearate were obtained from Amersham (Arlington Heights, IL). Sodium salts of fatty acids (palmitate, stearate, oleate, linoleate and linolenate) and glycerol lipid standards (triolein, diolein, and monoolein) were purchased from NuChek Prep (Elysian, MN). Sodium L-α-phosphatidate, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), D-glucose, Triton X-100 and 2,4,6-collidine were purchased from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid (TCA) and Scinti-Verse II were from Fisher Scientific (Fairlawn, NJ), and osmium tetraoxide (OsO_4) was from J.T. Baker Chemical (Phillipsburg, NJ). All other chemicals used were reagent grade of high purity and available from commercial sources.

Animals. Twelve Angus cattle (6 steers and 6 heifers) of the same age group (16–18 months) were selected from a single commercial Angus herd and finished with a standard finishing diet (16). The Angus breed was used in this study because of their propensity to develop more intramuscular adipose tissue in the longissimus dorsi muscle than most breeds. After reaching market weight (approximately 440 kg), the cattle were transported to the Texas A&M University Beef Cattle Center approximately one week prior to slaughter. Unusual stress was minimized during transportation and relocation of the animals. Upon arrival, cattle were assigned randomly and divided equally into two groups. One group of the cattle (3 steers and 3

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Abbreviations: BHT, 2,6-di-*tert*-butyl-4-methylphenol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TCA, trichloroacetic acid.

heifers) was subjected to a 72-hr fasting period in the holding pen with free access to water prior to slaughter, whereas the other group, fed *ad libitum*, was slaughtered about 4 hr after removal from feed. Carcass characteristics were evaluated according to USDA grading standards (17) by trained Texas A&M University personnel.

Tissue preparation. Immediately after stunning and prior to the completion of bleeding, the 8–13th rib region of the longissimus dorsi muscle from the right side of the animal was removed (with subcutaneous adipose tissue attached) by cutting through the hide. The sample was placed in 37°C Krebs-Henseleit calcium (Ca^{+2})-free bicarbonate buffer (pH 7.4) with 5 mM glucose, gassed with oxygen/carbon dioxide (95:5; v/v) for 30 min. Intramuscular and inner layer subcutaneous (adjacent to the muscle) adipose tissue snips were carefully dissected from the longissimus dorsi muscle for tissue incubation and adipose tissue cellularity determinations (1).

Tissue incubations. The incubation procedures and medium as described by Deeth and Christie (18) were modified for use in this study. Maximum weight of the adipose tissue snips in the incubation was limited to 150 mg (2–3 mm thickness) to minimize factors which tend to reduce fatty acid uptake (19). Adipose tissue snips (weight ranged from 70 to 150 mg) were incubated in Krebs-Henseleit Ca^{+2}-free bicarbonate buffer (pH 7.4) that contained 10 mM HEPES, 5 mM glucose, 1.0 mU/mL insulin, and 40 mg fatty acid-free bovine serum albumin for each μmol fatty acid. The fatty acid to bovine serum albumin molar ratio of 1.7 (molecular weight of albumin assumed to be 68,000) was established in accordance with a previous report (20). The sodium salt of the free fatty acid palmitate (16:0), stearate (18:0), oleate (18:1n-9), linoleate (18:2n-6), or α-linolenate (18:3n-3) was emulsified into the buffer by 30 min stirring at 37°C (for oleate, linoleate and linolenate) or 45°C (for palmitate and stearate) under nitrogen gas followed by 30 min sonication using a Fisher Sonic Dismembrator (Model 300, Fisher Scientific) equipped with a micro-tip on setting 3.5.

As reported by Pothoven and Beitz (11), and Bell (21), plasma free fatty acid levels can fluctuate from 0.3 mM to 2.0 mM. Therefore, three different fatty acid concentrations (0.3, 0.6 and 2.0 mM) were used in this study to determine the fatty acid esterification capacity of the adipose tissues. In addition, the concentrations of the exogenous fatty acid added were sufficient to minimize the effects of any endogenous unesterified fatty acids in the tissues. After the fatty acids were dispersed, the incubation media were gassed with O_2/CO_2 (95:5, v/v) for 30 min. Incubations were carried out in 20-mL glass scintillation vials, in duplicate, in 3 mL incubation medium (pH 7.4) containing 0.5 μCi of [9,10(n)-^{3}H]palmitate, [1-^{14}C]stearate, [9,10(n)-^{3}H]oleate, [1-^{14}C]linoleate, or [1-^{14}C]linolenate.

After gassing vials with O_2/CO_2 for 30 sec and sealing with caps, incubations were conducted for 30 min at 37°C in a shaking water bath (Fisher Model 129) set at 120 strokes/min (20). Five mL 5% (w/w) TCA was added to each vial to terminate incubations. Blanks included for all incubations were terminated by addition of 5 mL 5% TCA before incubation. Tissues were removed from the incubation media and were rinsed once with 9 mL incubation buffer and twice with 9 mL saline (0.154 M NaCl). The snips were transferred to scintillation vials containing 5 mL chloroform/methanol (2:1, v/v). To minimize lipid oxidation, 0.1 mg/mL of 2,6-di-*tert*-butyl-4-methylphenol (BHT) was added to the extraction solvent. The vials were flushed with nitrogen gas and were cap-sealed and stored at −25°C for subsequent lipid extraction and analysis.

Adipose tissue lipids were extracted by a method modified from Folch *et al.* (22). Nonesterified fatty acids and protein residues were trapped and removed with consecutive 0.38 M Na_2CO_3 washes (20). Preliminary tests showed that the Na_2CO_3 washes removed more than 99.7% of nonesterified fatty acids from the lipid extract. The chloroform was evaporated under a nitrogen gas stream at 45°C in a Multi-Vap (Model 112) evaporator. The dried lipid samples were redissolved in 0.3 mL chloroform for subsequent lipid assays.

The esterified lipid products were characterized and quantitated by thin-layer chromatography as described by Christie (23). The plates (preadsorbent/scored/channeled 20 × 20 cm, 250 micron Silica Gel G, Analtech, Inc., Newark, DE) were activated at 12°C for 2 hr and cooled to ambient temperature in a vacuum desiccator before sample applications. An unlabeled carrier standard (10 μL) consisting of a mixture of diolein, phosphatidate, and nonesterified oleic acid was applied to the sample spots. The plates were developed in a hexane/diethyl ether/glacial acetic acid (85:25:2.5, v/v/v) solvent system. Separated lipids were visualized and identified by comparison to migration of reference standards in parallel channels after exposure to iodine vapors. Respective bands of triglycerides, diglycerides, monoglycerides and phospholipids for each sample were scraped into 6-mL plastic scintillation vials (Beckman, Fullerton, CA) and, after adding 5 mL scintillation fluid, lipid products were quantified by liquid scintillation spectrometry. Esterification rates of fatty acids were calculated on the basis of incubation medium specific activity and were expressed as nmol fatty acid esterified/10^5 adipose tissue cells/30 min.

Adipose tissue cellularity. Subcutaneous and intramuscular adipose tissue samples (90 to 120 mg) were stored at −25°C in screw cap vials. Frozen tissue samples were sliced to approximately 1 mm thickness on an ice-cold glass plate and transferred into 20 mL scintillation vials. The method modified by Etherton *et al.* (24) was used for determining adipose tissue cellularity (cell size and cells per g adipose tissue).

Mean adipocyte diameter was obtained by randomly measuring 150 fixed cells for each tissue sample using a Timbrell/Coulter Shearicon (25). Adipocyte volume data were obtained by calculation based on cell diameter. The number of adipocytes per unit adipose tissue weight (g) was measured using a Coulter Counter (Model ZBI). Mean adipocyte number for each sample was obtained from five readings.

Statistical analyses. Data were processed and analyzed using the Statistical Analysis System (SAS) program (26). Main effects (independent variables) included sex (steers and heifers), treatments (fed and fasted), adipose tissues (subcutaneous and intramuscular) from each animal, fatty acids (palmitate, stearate, oleate, linoleate and linolenate) for each tissue, and fatty acid concentrations (0.3 mM, 0.6 mM and 2.0 mM). Because these were split-split plot designs, appropriate error terms were used to test the respective main effects and their interactions. The Student-Newman-Keul's test was performed to compare the mean differences when the main effect was significant.

RESULTS AND DISCUSSION

Adipose tissue cellularity. In agreement with previous reports (1-3,27,28), subcutaneous adipocytes were larger ($P<0.05$) than intramuscular adipocytes (Table 1). This investigation was initially designed to characterize differences in fatty acid esterification between steers and heifers. However, sex of the animal had no effect on any aspect of adipose tissue cellularity (Table 1) or fatty acid esterification (data not shown). Nor was dietary restriction sufficient to affect adipose tissue cellularity or rates or patterns of fatty acid esterification ($P>0.10$). Similarly, no interactions ($P>0.10$) among the main effects, sex (steer and heifer), treatment (fasted and fed) and adipose tissue (intramuscular and subcutaneous adipose tissues) were detected for all aspects of adipose tissue cellularity. For this reason, only differences elicited by media fatty acid concentration and adipose tissue depot will be discussed.

Esterification as a function of fatty acid concentration. Fatty acid incorporation into the adipose tissue total lipids increased with fatty acid concentration (Fig. 1). With the exception of stearate, the increase in esterification generally was linear with fatty acid concentration up to 2.0 mM. This is similar to previous findings (20,29). The variability in rates at different concentrations of stearate may have been due to extreme insolubility of stearate in the buffer system. It is unlikely that this abnormality was caused by the detergent effect of the fatty acid (30), because the greatest rate of esterification for stearate was observed at 2.0 mM.

At the low concentrations of substrate fatty acids, it is possible that lipolysis could have diluted the intracellular pool of radioisotopically-labeled fatty acids. This would have been especially pronounced in the cattle fasted for two days. However, we observed no difference in rates of esterification of fatty acids between the fed and fasted steers (data not shown), suggesting that fatty acid pool dilution did not substantially affect the observed rates of incorporation. Virtually no effect of pool dilution would be anticipated for tissues incubated at 2.0 mM fatty acids, or for those fatty acids in low abundance in bovine adipose tissues (linoleate and α-linolenate; ref. 31).

For adipose tissues incubated with palmitate, oleate and linolenate, the triglyceride fraction decreased as the fatty acid concentration increased (from 80-85% of the total to 70-75% of the total; data not shown). This was generally accompanied with a relative increase in the percentage of diglyceride, phospholipid and monoglyceride fractions. The distribution of stearate and linoleate in glycerolipid fractions did not change with substrate concentration. A decrease in the proportion of triglycerides with increased diglycerides and phospholipids was also noted in ovine liver slices incubated with increasing concentrations of various fatty acids (32). The basis for the alteration of lipid distribution in response to the medium fatty acid concentration is not known.

Fatty acid esterification as a function of fatty acid species. To characterize the effects of the different exogenous fatty acids on esterification rates and lipid product distributions, data were pooled across fatty acid concentrations (Table 2). Palmitate, followed by oleate, were esterified more rapidly ($P<0.05$) than linoleate, linolenate and stearate by the adipose tissues *in vitro*. The incorporation rate tended to be higher for linolenate than for linoleate, though the difference ($P<0.10$) was noted only in subcutaneous adipose tissue (Table 2). Stearate had the lowest esterification rate among the five fatty acids. Similar results were observed in ovine adipose tissue (32), but the esterification rate of stearate was much higher than reported here. Another study (18) demonstrated that oleate, palmitate and stearate were esterified at similar rates when ovine adipose tissue was incubated with equimolar concentrations of the fatty acids. The discrepancy among studies may be due to differences in the fatty acid makeup of the incubation medium. Since fatty acids are stereospecifically esterified onto the glycerolipids (7,30, 33), incubation with mixtures of fatty acids would be more favorable for incorporation than with homogeneous fatty

TABLE 1

Intramuscular and Subcutaneous Adipose Tissue Cellularity of Fed and Fasted Steers and Heifers[a]

	Sex/treatment				Comparison within adipose tissue	
	Heifer		Steer			
Measure	Fed	Fasted	Fed	Fasted	Fed *vs.* fasted	Steer *vs.* heifer
			Intramuscular adipose tissue			
Cells/g adipose tissue[b] $\times 10^{-5}$	4.79 ± 1.25	3.09 ± 0.56	5.22 ± 0.96	4.27 ± 1.29	NS[d]	NS
Cell volume[c] $\mu m^3 \times 10^{-5}$	12.00 ± 3.21	12.18 ± 2.44	12.35 ± 1.03	13.04 ± 2.25	NS	NS
			Subcutaneous adipose tissue			
Cells/g adipose tissue[b] $\times 10^{-5}$	2.69 ± 0.15	3.78 ± 1.26	3.77 ± 0.68	2.49 ± 0.17	NS	NS
Cell volume[c] $\mu m^3 \times 10^{-5}$	23.60 ± 2.68	29.67 ± 6.48	27.60 ± 4.65	26.22 ± 2.69	NS	NS

[a] Adipose tissues were fixed by 3% (w/v) OsO_4 (in collidine buffer) and adipocytes were isolated in 8 M urea. The number of adipocytes per unit adipose tissue weight (g) was measured using a Coulter Counter (Model ZBI) equipped with a logarithmic range expander. Adipocyte diameter (d) for each tissue sample was obtained by randomly measuring 150 fixed cells using a Timbrell/Coulter Shearicon. Adipocyte volumes were obtained by calculation $[4/3\pi(d/2)^2]$ from the cell diameter data for each tissue sample. Values are expressed as mean ± SEM.

[b] Cells/g adipose tissue were not different ($P>0.05$) between intramuscular and subcutaneous adipose tissues.

[c] Cell volume was different ($P<0.05$) between intramuscular and subcutaneous adipose tissues.

[d] Not significant.

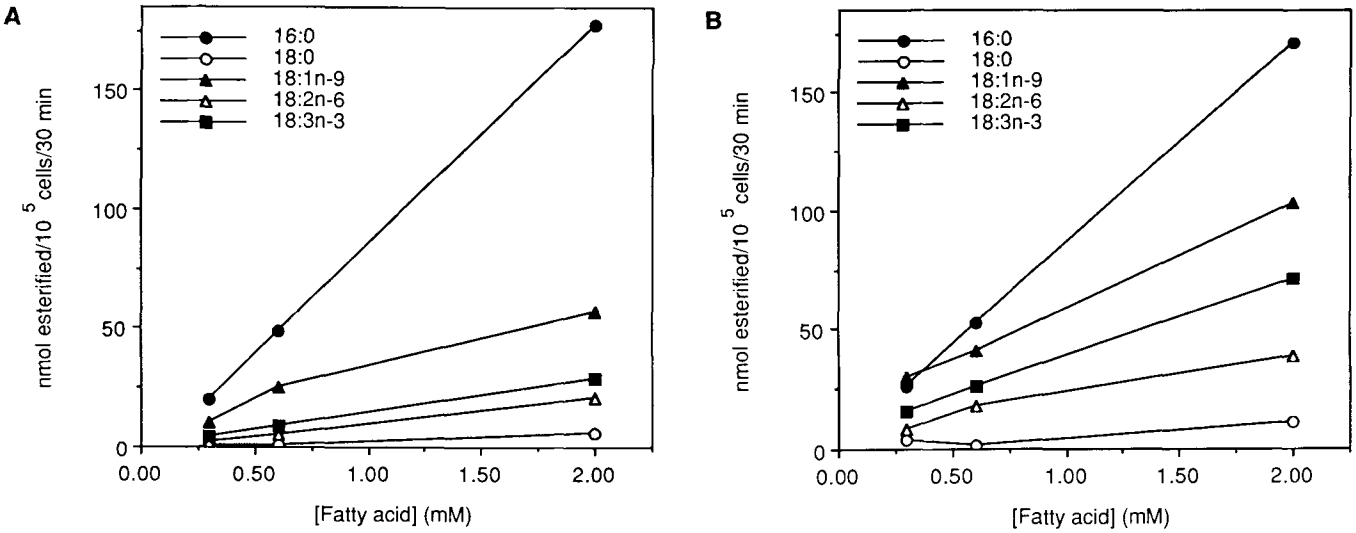

FIG. 1. The esterification of palmitate (16:0), stearate (18:0), oleate (18:1n-9), linoleate (18:2n-6) and α-linolenate (18:3n-3) into total lipids in bovine intramuscular adipose (A) and subcutaneous (B) adipose tissues incubated *in vitro*. Each data point is the mean of adipose tissues obtained from six steers and six heifers. The average SEM was 23% of the mean.

TABLE 2

Fatty Acid Esterification Rates and Percentage Distributions in Glyceride Fractions in Intramuscular (IM) and Subcutaneous (SC) Adipose Tissues Incubated with Various Fatty Acids[a]

Lipid	Adipose tissue	Fatty acid				
		16:0	18:0	18:1	18:2	18:3
		(nmol fatty acid esterified/10^5 cells/30 min)				
Total lipids	IM	82.36 ± 16.36^{b}	2.53 ± 0.47^{c,d}	30.83 ± 5.88^{e,d}	9.52 ± 1.99^{c,d}	14.02 ± 3.37^{c,d}
	SC	83.11 ± 13.20^{b}	5.41 ± 1.00^{f}	57.53 ± 7.54^{e}	21.21 ± 3.09^{g}	37.35 ± 7.90^{c}
		(% of total lipid esterified)				
Triglyceride	IM	85.01 ± 1.40^{b,d}	64.75 ± 3.29^{e,d}	72.45 ± 3.65^{e}	64.30 ± 3.63^{e}	70.14 ± 3.25^{e,d}
	SC	75.46 ± 1.74^{b}	74.52 ± 2.53^{b}	72.29 ± 2.97^{b}	72.87 ± 3.65^{b}	80.40 ± 2.04^{b}
Diglyceride	IM	6.87 ± 0.62^{e,d}	18.81 ± 2.11^{b}	15.57 ± 2.26^{b}	15.34 ± 2.36^{b,d}	13.27 ± 1.30^{b,d}
	SC	13.70 ± 1.38^{b}	13.22 ± 1.33^{b}	12.40 ± 1.58^{b}	11.06 ± 1.30^{b}	9.20 ± 0.92^{b}
Monoglyceride	IM	4.87 ± 1.10^{b}	7.73 ± 1.21^{b}	5.74 ± 1.14^{b}	8.58 ± 1.55^{b}	7.48 ± 1.32^{b,d}
	SC	4.87 ± 0.69^{b}	5.95 ± 1.15^{b}	7.05 ± 0.96^{b}	8.19 ± 2.79^{b}	4.48 ± 0.83^{b}
Phospholipid	IM	3.25 ± 0.56^{c,d}	8.70 ± 1.25^{b,e,d}	6.24 ± 1.39^{e,c}	11.78 ± 2.29^{b}	9.11 ± 1.42^{b,e,d}
	SC	5.97 ± 0.80^{b}	6.31 ± 0.76^{b}	8.27 ± 1.32^{b}	7.87 ± 2.77^{b}	5.93 ± 1.10^{b}

[a]Adipose tissue snips (70–150 mg) were incubated in 3.0 mL of Krebs-Henseleit Ca^{2+}-free bicarbonate buffer containing 10 mM HEPES, 5 mM glucose, 1.0 mU/mL insulin, fatty acid-free bovine serum albumin, fatty acid (palmitate, stearate, oleate, linoleate or linolenate) and 0.5 μCi of the labeled fatty acid. Data are pooled across fatty acid concentration. Means ± SEM are for three steers and three heifers with three concentrations fatty acid/animal.

Means within rows followed by different superscript letters (b,c,e,f,g) are different ($P<0.10$); means between the intramuscular (IM) and subcutaneous (SC) adipose tissues (d) are different ($P<0.10$).

acid media, as was used in the present investigation. The differences between the current study and previous investigations of ovine adipose tissue alternatively may reflect species differences. We have demonstrated that ovine and bovine subcutaneous adipose tissues differ in several aspects of *de novo* fatty acid biosynthesis (34).

Rates of esterification of stearate, oleate, linoleate and linolenate into triglycerides were higher ($P<0.10$) in subcutaneous adipose tissue than in intramuscular adipose tissue (calculated from data in Table 2). Conversely, the rate of palmitate incorporation into triglycerides was the same in intramuscular and subcutaneous adipose tissues. Regardless of the fatty acid substrate, subcutaneous adipose tissue consistently exhibited greater ($P<0.10$) rates of diglyceride production than did intramuscular adipose tissue. A similar trend was observed for phospholipids, although the differences were significant only for palmitate and oleate. The only differences ($P < 0.10$) noticed in

monoglycerides between the two adipose tissues were for oleate and linoleate.

The rates of fatty acid incorporation into total lipids (Table 2) were expressed per 10^5 adipocytes (*i.e.*, per metabolic unit). However, the volume of the subcutaneous adipocytes was over twice that of the intramuscular adipocytes (Table 1). If rates were expressed based on adipocyte volume, then the esterification of palmitate in intramuscular adipocytes would be over two-fold greater than the rate observed in subcutaneous adipocytes. This indicates that the enzymes of the triglyceride biosynthetic pathway are relatively more concentrated in the intramuscular adipocytes than in their subcutaneous counterparts.

The different rates of palmitate and linoleate esterification in the current study reflect the natural composition of these fatty acids in bovine adipose tissues (7,35,36). However, because ruminant animals biohydrogenate fatty acids before passage into the small intestine, very little linoleate is available for absorption. Thus, fatty acid availability, rather than rate of esterification, likely dictates the composition of bovine adipose tissues for many of the fatty acids.

There is no clear explanation for the higher oleate content (42–49% *vs.* 25–30% for palmitate) but lower esterification rate of oleate as compared to palmitate. It was reported that oleoyl-CoA at a high concentration (0.12–0.27 mM) reduced the activity of phosphatidate phosphohydrolase (37). The high concentration of oleate in bovine adipose tissue, relative to palmitate, may also reflect the active fatty acid elongase and stearoyl-CoA desaturase of that tissue (38), so that a large proportion of the palmitate synthesized *de novo* or absorbed from the diet would be converted to oleate.

With respect to the low stearate incorporation rate, the difficulty in dispersing the water-insoluble substrate as well as maintaining its emulsion stability, in part, could be the reason for both its low incorporation rate *in vitro* and the relatively lower concentration observed in bovine adipose tissue (39). Fatty acyl-CoA synthetase (EC 6.2.1.3) has a low affinity for stearate relative to its affinity for the other fatty acids (40). Stokes *et al.* (41) demonstrated that stearoyl-CoA was incorporated into triglycerides by the subcellular preparations from rat adipose tissue as efficiently as palmitoyl-CoA, but with stearate the incorporation rate was only about one-fifth that of palmitate, oleate and linoleate. On the basis of these observations, they concluded that this difference was at the level of acyl-CoA synthetase (41).

Fatty acid distribution in glycerolipids as a function of fatty acid species. Irrespective of the fatty acid species, triglycerides were the predominant products and amounted to 60–85% of total lipids esterified (Table 2). The subsidiary lipid class (9–20%) formed by the adipose tissue was diglycerides, and the remainder was accounted for by monoglycerides and phospholipids. Deeth and Christie (18) and Payne and Masters (32) reported similar findings in ovine omental adipose tissue incubated with the same fatty acids.

For stearate, linoleate and linolenate, subcutaneous adipose tissue tended to have a larger proportion of triglycerides but smaller proportions of diglycerides and phospholipids than did intramuscular adipose tissue, though the differences were not always significant (Table 2). In contrast, intramuscular adipose tissue deposited a larger proportion of palmitate as triglyceride than did subcutaneous adipose tissue. There were no differences ($P > 0.10$) in the distribution of oleate into these lipid products between the two adipose tissues. Except for linolenate, the variation in monoglyceride distributions in the two adipose tissues was not significant ($P > 0.10$).

The lipid products were distributed in a pattern parallel to the natural distribution of the lipid classes in adipose tissue, with triglycerides being the primary products followed, in descending order, by diglycerides, phospholipids and monoglycerides (9). Because the major role of adipose tissues is to assimilate and deposit fatty acids as triglycerides, it may be expected that the adipose tissues primarily synthesize triglycerides *in vitro*. The apparent accumulation of diglycerides could be an artifact arising from the time lag in the formation of triglycerides from diglycerides.

In summary, the different esterification activities between intramuscular and subcutaneous adipose tissues support the previous findings that adipose tissues from various anatomical origins are dissimilar in metabolic functions and processes (1–3,42,43). It has been shown that intramuscular adipose tissue can synthesize palmitate *in situ*, but at a much slower rate than subcutaneous adipose tissue (1,43,44). Because of its low endogenous synthetic capacity, intramuscular adipose tissue may rely more on exogenous sources of palmitate to supply substrate for glycerolipid assimilation than subcutaneous adipose tissue.

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Anorexic Contribution to Increased Linoleate Mobilization and Oxidation in Lymphoma-Bearing Mice

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To test for a regulatory defect in adipose triacylglycerol (essential) fatty acid mobilization in lymphoma-bearing mice, free [1-^{14}C]linoleic acid/mouse serum albumin was injected *iv* into lymphoma-bearing and control mice, adapted to a reversed light cycle, and studied in three dietary states in the dark period. Mean daily food intake decreased in mice with small and large tumor burdens. Plasma free fatty acid (FFA) oxidation rates, which approximate FFA mobilization rates, were estimated by multicompartmental analysis (CONSAM). Oxidation of linoleate to CO_2 was reduced significantly (85%) in *ad libitum* fed as compared to briefly fasted control mice but not in fed *vs.* fasted mice with large or small tumor burdens. However, plasma FFA oxidation rates to CO_2 did not differ in briefly fasted tumor-bearing and pair-fed control mice. When re-fed a 250-mg test meal, briefly fasted mice with small tumors suppressed plasma free linoleic acid oxidation, as did controls. During simulated night, mildly anorexic, tumor-bearing mice with small tumor burdens mobilized essential fatty acids much faster than controls. This could explain body fat loss. The abnormally rapid rates of FFA (free linoleic acid) mobilization at night probably result from anorexia rather than from inability of food to suppress fat mobilization.
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Lymphoma-bearing mice reportedly have a potent, circulating lipid mobilizing factor (LMF) produced directly by the lymphoma cells. This factor was hypothesized to act by increasing the mobilization of linoleate, and presumably other fatty acids, stored in adipose tissue as triacylglycerol fatty acids (TGFA) (1,2). Such action could explain the loss of body fat, an aspect of cancer cachexia associated with this mouse lymphoma (1), and also enhance tumor growth, which is limited by the free linoleic acid transport rate in some experimental cancer models (3). However, no one, to our knowledge, has measured transport rates of free fatty acid (FFA), traced by free linoleic acid, in lymphoma-bearing mice to confirm that mobilization of this essential FA is actually increased.

Two studies of non-essential FA have been carried out in lymphoma-bearing mice, both of which failed to show any increase in FFA (free palmitic acid) mobilization and oxidation in briefly fasted lymphoma-bearing mice (4,5). Therefore, if an active LMF circulates in the lymphoma-bearing mice, it does not influence rates of adipose TGFA lipolysis in the fasted state. However, free palmitic acid is mobilized and oxidized more rapidly in *ad libitum* fed lymphoma-bearing mice than in controls, suggesting that LMF, if present, might interfere with regulatory processes that govern lipolytic activity in adipose tissue (5). Alternatively, diminished food intake, independent of LMF activity, could account for the abnormally rapid rates of FFA (free palmitic acid) mobilization in fed lymphoma-bearing mice (5).

In the present study we have used tracer techniques to test the hypothesis that FFA (free linoleic acid) transport and oxidation is increased in lymphoma-bearing mice, presumably due to the presence of a potent, circulating LMF. We have also monitored food intake throughout the tumor-growth period, as well as before and after adaptation to the reverse light cycle conditions under which the studies were carried out, in order to establish whether the hypothetical defect in essential FFA transport might result from anorexia. Finally, we have included a set of experiments designed to see whether lymphoma-bearing mice are capable of responding to a known small test meal by reducing their rates of FFA (free linoleic acid) transport and oxidation as do control mice.

MATERIALS AND METHODS

Materials. [1-^{14}C]Linoleic acid (58 mCi/mmol) was purchased from ICN Biochemicals, Inc. (Costa Mesa, CA) and was 99% pure. Aquasol was obtained from Dupont-NEN (Boston, MA). All solvents and chemicals used in CO_2 and FFA measurements were of reagent grade.

Animals and tumors. Seven-week-old male AKR/J mice (Jackson Laboratory, Bar Harbor, ME) were maintained on Purina Lab Chow and water *ad libitum*. The mice were divided into lymphoma-bearing and control groups. Mice in the tumor-bearing (TB) group were inoculated with 1×10^6 freshly harvested SL-3 tumor cells that had been maintained in culture (6). The cells were injected *ip* in 0.2 mL medium or phosphate buffer (5). The tumor usually developed in about two weeks and was detected by a palpable abdominal mass near the inoculation site and by decreased food intake by the animal.

Both control and TB mice were maintained on a reverse light cycle schedule which consisted of 12 hr of darkness (8:00 a.m.–8:00 p.m.), followed by 12 hr of light at night. Mice from both groups were kept on a reverse light cycle immediately after the tumor inoculation and were maintained under the same conditions throughout the study, but with additional minimal lighting required to inject the tracer and to execute the experiment.

Animals were housed individually and body weights and daily food intake were monitored in all but one study, as described previously (5). The TB mice were studied at two levels of tumor burden, "small" and "large" (see

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Abbreviations: EAT, Ehrlich ascites tumor; EFA, essential fatty acid(s); FCR, fractional catabolic rate; FFA, free fatty acid(s); I.D., injected dose; LMF, lipid mobilizing factor(s); SAAM/CONSAM, computer programs used for simulation analysis and modeling; TB, tumor-bearing; TGFA, triacylglycerol fatty acid(s); TNF, tumor necrosis factor; VLDL, very low density lipoprotein.

Results for further description of the body weights, tumor sizes and average daily food intakes).

Experimental protocols. Four experiments listed below were carried out on separate populations of mice. Each study was conducted in the dark period after the mice had been adapted to a reversed light cycle for 2–3 weeks, the time allowed for tumor development. From these studies we were able to estimate the rates of plasma FFA transport and oxidation to CO_2, based on free linoleic acid as tracer, in the following—lymphoma-bearing animals with small or with large tumor burdens and their controls, fed *ad libitum;* tumor bearing (small burdens) and pair-fed controls in both the briefly fed state and after ingesting a small, known test meal.

In Experiments 1 (controls) and 2 (lymphoma-bearing mice with small burdens), free [1-^{14}C]linoleic acid disappearance from plasma and oxidation to breath $^{14}CO_2$ in unanesthetized mice that were fed *ad libitum* was measured. Fatty acid/mouse serum albumin complexes for all experiments were prepared as described previously (7). Each experiment was conducted in two parts, on consecutive days, at mid-day. The dose (1 μCi/mouse) in 0.2 mL of mouse serum was injected into the tail vein of unanesthetized mice in restraining cages. On the first day, only breath $^{14}CO_2$ was measured. On the second day, the same animals were used to measure plasma [^{14}C]FFA disappearance by collecting two retroorbital blood samples per mouse, from half of the mice at 0.5 and 2 min, and from the other half at 1 and 5 min plus the terminal sample at 10 min from all control and from TB mice with small burdens. The numbers of mice used in Experiments 1 and 2 were 9 and 8, respectively.

In Experiment 3, TB mice with large tumor burdens were studied under the same conditions as in Experiments 1 and 2, except that they were only injected with tracer once to study the kinetics of breath $^{14}CO_2$ appearance for 10 min. Immediately thereafter a terminal blood sample (only) was drawn to determine the percent of the injected [^{14}C]FFA dose remaining in the plasma.

In Experiment 4, we again measured the oxidation of plasma free [1-^{14}C]linoleic acid oxidation to CO_2 following *i.v.* injection of the tracer in control and in TB mice (small burdens), and the percentage of the injected tracer remaining in the plasma at 10–11 min. However, in this experiment the control mice were carefully pair-fed with the TB animals, and a different experimental protocol was followed. All of the mice were briefly fasted and then half were fed a single 250-mg test meal for 30 min immediately prior to the tracer study, and half were not fed. Four groups of mice (n=5/group) were used: pair-fed controls, briefly fasted, 4–6 hr; TB, briefly fasted; pair-fed controls, briefly fasted and then refed; and TB mice, briefly fasted and refed. All studies were carried out in the dark period, beginning at noon, as in the other experiments. Only a terminal blood sample, at 10–11 min, was collected immediately after the last breath $^{14}CO_2$ sample. Plasma FFA data (percent injected dose remaining and plasma FFA concentration) were obtained from this terminal sample. The mixing kinetics of plasma with extraplasma FFA were assumed to be the same as those of the corresponding *ad libitum* fed group.

Blood sampling. Retroorbital blood was obtained from an opthalmic venous sinus in heparinized capillaries (8,9). Only two blood samples per mouse (100 μL total volume) were taken prior to a third terminal sample. The capillaries were kept in ice and then centrifuged at 1,000 rpm for 1 min to separate plasma from red blood cells.

Chemical and radioactive analyses. Plasma [^{14}C]FFA was determined by adding 25–100 μL of plasma to 10 mL Aquasol and counting directly, without prior separation of FFA from other lipids. We confirmed in trial runs that the serum total neutral lipid radioactivity in the short time span (10 min) used in these experiments is entirely in the form of labeled FFA. Plasma FFA concentrations were determined using terminal blood samples according to the method of Hron and Menahan (10), as in our earlier studies (4,5,11). Total plasma [^{14}C]FFA radioactivity at each time was calculated by assuming a plasma volume of 4.0% of body weight (4).

Breath CO_2 measurement. The techniques used for measuring radioactivity and mass of CO_2 were those described in our earlier studies (5,12), namely, trapping in hyamine hydroxide solution, measuring the time needed for the expired CO_2 to titrate the latter to a phenolphthalein end point (12), and measurement of bicarbonate radioactivity by liquid scintillation counting (0.5 mL of the trapping agent in 10 mL Aquasol). Breath $^{14}CO_2$ was collected for 10 min, with measurements of radioactivity every 2 min, cumulatively.

Multicompartmental analyses. The tracer kinetic data that were analyzed consisted of the percentage of the injected dose of radioactive free linoleic acid remaining in the plasma as a function of time and the percentage of the injected dose appearing in breath $^{14}CO_2$ cumulatively during the first 10 min of the experiment. The disappearance of labeled FFA from the plasma has been shown in earlier studies (11) to represent primarily mixing of the tracer with extraplasma FFA (and FA-X) compartments. Only a part of the label appears in the breath during this period. The multicompartmental analysis, as indicated below, takes these complex mixing and metabolic processes into account.

Multicompartmental analyses were carried out using the SAAM and CONSAM programs (13,14) with a VAX 11-780 computer using the model shown in Figure 1 of Kannan *et al.* (5). The reader is referred to the latter study and to our previous studies (11) for the logic and assumptions used in developing and validating the model, as well as for data and parameters that define the mixing and turnover of the plasma and extraplasma bicarbonate/CO_2 compartments in TB and in control mice (5). We assumed here that the parameters obtained using CONSAM for the bicarbonate/CO_2 compartments of briefly fasted control and TB mice studied in the regular light cycle could be applied to the corresponding control and TB mice under all of the present conditions. Although the bicarbonate kinetics almost surely differ in the different dietary states, this should have no major impact on our estimates of the fractional rate of plasma FFA oxidation to CO_2, the parameter used to test our hypotheses.

Statistical analysis. The significance of the differences between mean values for any fractional rate constant or transport rate was assessed by constructing an approximate 95% confidence interval, as described earlier (5). Differences between means of plasma FFA pool sizes and of [^{14}C]FFA remaining in the plasma at 10 min were evaluated using Student's *t*-test.

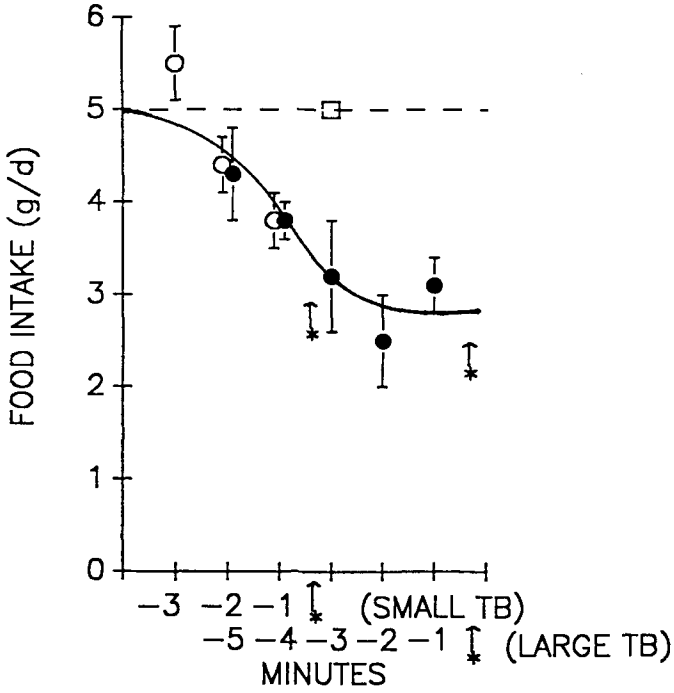

FIG. 1. Mean daily food intakes of lymphoma-bearing mice with small (○) and large (●) tumor burdens during the period immediately preceding the tracer studies. The mornings of the separate tracer studies (*i.e.*, during day 0) are indicated for each TB group by an arrow and asterisk. Separate populations of mice were used for each study. Data (□), broken line as reference, are also shown for control mice, some of which were later used for pair-feeding with the TB mice with small burdens, but before pair-feeding commenced. All mice were fed *ad libitum* and are the means of 8, 9, and 19 mice for the following groups, small tumors, large tumors, and controls, respectively. Food intakes have been normalized to 27 g body weight.

RESULTS

Mean body weights, food intake, inguinal fat pad, and tumor wet weights in control and lymphoma-bearing mice. Body weights of *ad libitum* fed mice on the morning of the linoleate oxidation studies averaged (± SE) 24.5 ± 0.52, 23.3 ± 1.9, and 26.9 ± 1.1 g for controls, mice with small tumor burdens, and mice with large tumor burdens, respectively; body weights were 29.5 ± 0.48 and 27.9 ± 0.70 g (fasted groups, mice with small tumor burdens, and pair fed controls, respectively) and 28.7 ± 0.89 and 24.0 ± 0.71 g (fasted-refed groups). Initial body weights, at tumor inoculation, when mice were first placed under reversed light cycle conditions averaged 25.4 and 22.6 g for the mice used in the fasted and fasted-refed studies, respectively.

Lymphoma-bearing mice with small burdens eat about the same amount of food as they do when first inoculated and when first placed on a reversed light cycle (5). However, during adjustment to the reversed light cycle, the daily food intake of both *ad libitum* fed controls and TB mice increases significantly; it then decreases in the TB mice, but not in the controls. This is shown in Figure 1. The food intake, normalized to 27 g body weight, the approximate mean body weight of all groups on the experimental day, increased from 4.3 ± 0.11 g/day in the first week of acclimatization (not shown in Fig. 1) to 5.0 ± 0.09 g/day ($p \leq 0.01$) three days prior to study (control mice). In Figure 1, day −1 represents food intake by mice with small and large tumor-burdens (and their pair-fed controls) from the day before the experiment to the day of the experiment. The mean daily food intake in TB mice with small tumor burdens fell significantly (from 5.0 or 5.5 to 3.6 g/day) during the three days prior to the experimental day (Fig. 1). Similarly, the mean food intake of mice with large tumor burdens fell during the five days preceding the experimental day. Thus, in mice with large tumor burdens, there was a 53% reduction in food intake (from 5.5 to 2.6 g/day) as compared to values measured six days earlier (Fig. 1). Pair-fed controls were offered and ate the same amount of food as the TB mice ate on the preceding day. Tracer studies of the pair-fed controls were done the day after those of TB mice so that pair-feeding could continue until the experimental day.

As in our earlier report (5), mice with small tumor burdens had solid *ip* tumors weighing less than 0.5 g and some gelatinous ascites material that was not quantified. Mice with large tumor burdens had an average of 2.5 ± 0.5 g solid tumor and their right inguinal fat pad weights were significantly lower than those of controls (20 ± 6 *vs.* 109 ± 12 mg/single fat pad, respectively). The inguinal fat pad weights of mice with small tumor burdens did not differ significantly from those of controls (5).

Plasma free [1-^{14}C]linoleic acid disappearance following i.v. *injection of tracer free linoleic acid/mouse serum albumin complex.* Early kinetics of plasma FFA (linoleic acid) mixing and removal during experiments of 10 min duration were studied in two groups of mice, those with small tumor burdens and controls, each group fed *ad libitum* (Fig. 2). The tracer remaining in plasma at 10 to

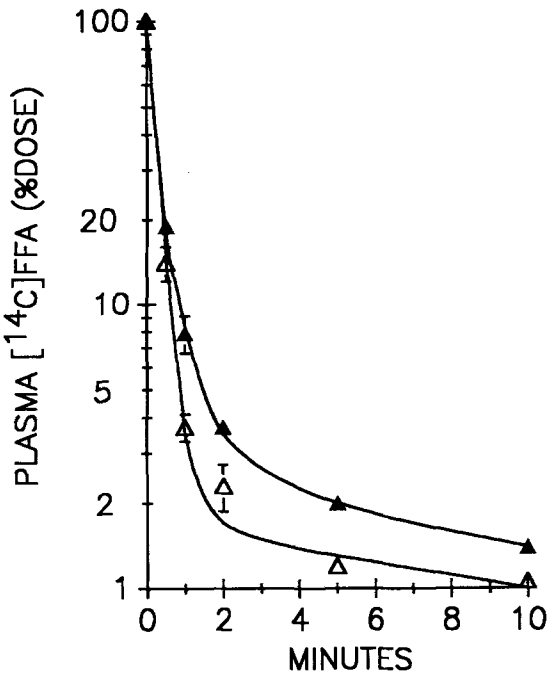

FIG. 2. Disappearance of plasma [^{14}C]FFA after *i.v.* injection of free [1-^{14}C]linoleic acid complexed to mouse serum albumin in lymphoma-bearing (▲) and control (△) mice fed *ad libitum*. Mice were adapted to a reversed light cycle and studied in the dark period. Ordinate: percent of injected ^{14}C dose remaining in the total plasma volume as FFA; data plotted semilogarithmically. Number of mice: controls, 9; tumor (small burdens), 8. Vertical bars, ± SE.

11 min, measured immediately after the studies of free [1-^{14}C]linoleic acid oxidation to breath $^{14}CO_2$ (below), was measured in each of the other groups. The percentage of the injected dose remaining in the plasma per mouse did not vary greatly among groups, and ranged [mean ± SE (n)] from 1.0 ± 0.06 (n=6) to 1.6 ± 0.015 (n=5) %. In all groups studied, an average of between 98.4% and 99.0% of the injected free linoleic acid dose was removed from the circulation in 10–11 min. This implies that mixing of plasma FFA with extraplasma FFA and with other rapidly exchanging FA-containing compartments, which dominates the early kinetic behavior of injected tracer FFA (11), occurred at similar rates in all of these groups. Accordingly, the early tracer FFA data between t = 0.5 and 5 min for the normal and TB mice fed *ad libitum* (shown in Figure 2) was assumed to approximate that of the normal and TB mice, respectively, in the other groups.

Kinetics of plasma free [1-^{14}C]linoleic acid oxidation to breath $^{14}CO_2$ in TB and control mice (reversed light cycles). In control mice fed *ad libitum,* the cumulative excretion of breath $^{14}CO_2$ was 85% lower than in those that were briefly fasted [6% *vs.* 30%, respectively (Fig. 3)]. However, oxidation of labeled linoleate to breath $^{14}CO_2$ was the same in the fed as in the briefly fasted lymphoma-bearing mice (Fig. 4). Thus, averages of 27% and of 30% of the injected dose were recovered as $^{14}CO_2$ at 10 min in *ad libitum*-fed TB mice with small and large tumor burdens, respectively (Fig. 4). This contrasts with the mean value of 6% for the *ad libitum*-fed control mice (Fig. 3), and is similar to the mean value of 30% for the briefly fasted TB mice with small tumors (Fig. 4).

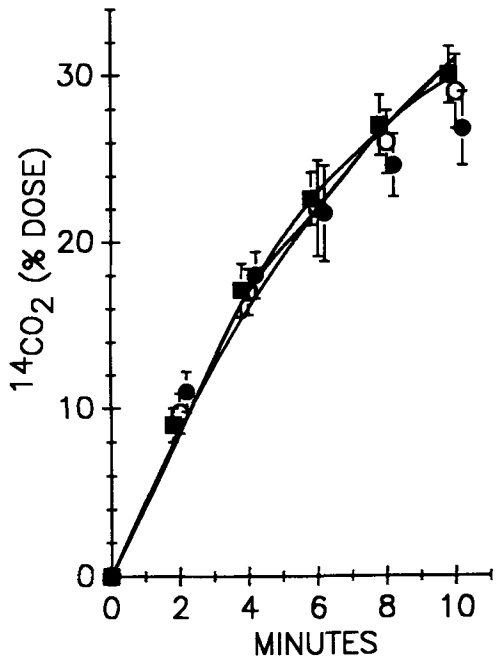

FIG. 4. Failure of lymphoma-bearing mice, both with large (○) and small (●) tumor burdens, to suppress plasma FFA (free linoleic acid) oxidation when fed *ad libitum,* compared to briefly fasted (■) TB mice with small tumor burdens (4–6 hr). See legend to Figure 3 for further details. Number of *ad libitum*-fed TB mice: small burden, 8; large burden, 8; number of fasted TB mice (small burden), 5. Vertical bars, ± SE.

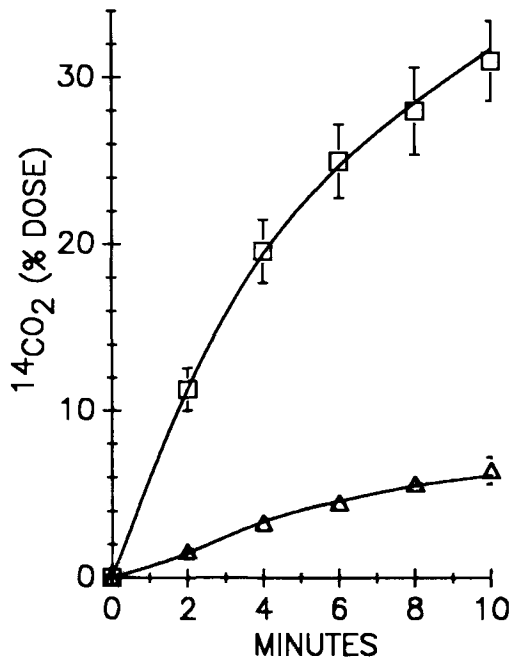

FIG. 3. Suppression of plasma FFA (linoleate) oxidation to CO_2 in *ad libitum*-fed (△) compared to briefly (4–6 hr) fasted (□) control mice. The cumulative breath $^{14}CO_2$ excretion patterns, expressed as the percentage of the injected dose, are shown for the two groups as a function of time following *i.v.* injection of tracer free [1-^{14}C]linoleic acid complexed to mouse serum albumin. Data are plotted on rectangular coordinates. Number of mice: fasted, 5; fed *ad libitum,* 9. Vertical bars, ± SE.

Although it would appear from the data presented in Figure 4 that the TB mice, even those with small tumor burdens, do not suppress the mobilization and oxidation of free linoleic acid when they eat, this was not the case when the study was repeated under conditions in which food intake was carefully controlled. As shown in Figure 5, when fed a small (250-mg) test meal, both TB (small tumor burdens) and control mice responded similarly with a prompt, significant reduction in the rate of injected tracer linoleate oxidation to breath $^{14}CO_2$. This suppression was accompanied by a significant decrease in the plasma FFA levels (Table 1).

Three control mice were pair-fed to TB mice with small tumor burdens throughout the period of adaptation to the reversed light cycle, which corresponded to the period of tumor growth. Since food intake decreased in the TB group as compared to controls fed *ad libitum* (Fig. 1), it was also reduced in the pair-fed control mice for several days. However, the pair-fed control mice were then allowed to eat *ad libitum* on the morning of the tracer experiment. These control mice oxidized free [1-^{14}C]linoleic acid to breath $^{14}CO_2$ at the same slow (suppressed) rate as did control mice that had not had their food restricted at any time [(mean ± SE) of 5.8 ± 1.2% *vs.* 6.4 ± 0.81% of the injected dose excreted as $^{14}CO_2$ in 10 min, respectively, unpublished observations and Fig. 3]. Therefore, we conclude that it is not the diminished food intake in the several days immediately preceding the tracer study, but rather the diminished food intake on the experimental morning (Fig. 1) that accounts for the *ad libitum*-fed TB mice having elevated rates of plasma essential and non-

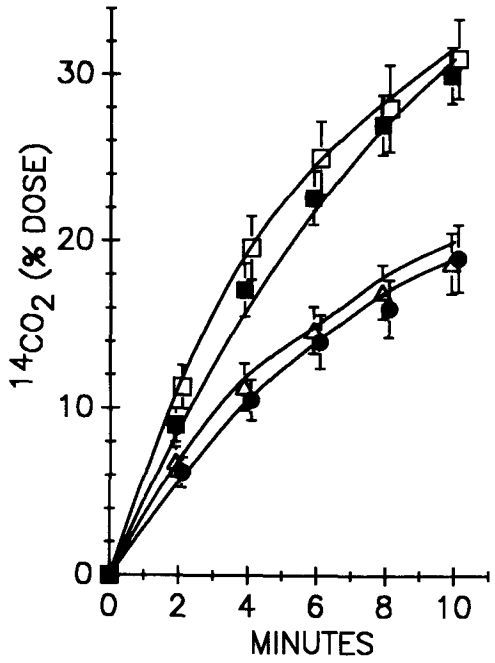

FIG. 5. Suppression of plasma FFA (free linoleic acid) oxidation to CO_2 in lymphoma-bearing and pair-fed control mice, briefly fasted and then fed a single, small test meal. Four groups of mice were used: briefly fasted, pair-fed controls (□); briefly fasted, pair-fed controls refed a 250-mg test meal (△); briefly fasted TB mice (■); and briefly fasted TB mice refed a 250-mg test meal (●). Data for the briefly fasted, pair-fed controls and TB mice are the same as those already presented in Figures 3 and 4. See legend to Figure 3 for further details. Number of mice, 5 per group. Vertical bars, ± SE.

essential (5) FFA oxidation and, therefore (11), elevated rates of adipose TGFA mobilization.

Multicompartmental analyses of the tracer (free linoleic acid) data. Plasma FFA pool sizes, the estimated fractional rates of plasma FFA oxidation to CO_2, and the plasma FFA oxidation rates (mass/time), which are assumed to approximate the net rates of plasma FFA replacement by mobilization of adipose TGFA to plasma FFA (11), are given in Table 1 and described in further detail below.

Control and TB mice fed ad libitum. The mean plasma FFA pool size of the *ad libitum*-mice with small tumor burdens was twice as high ($p \leq 0.001$) as that of the fed controls. The elevated plasma FFA pool sizes were associated with four-fold higher fractional rates of plasma FFA oxidation to CO_2 and nine-fold higher rates of plasma FFA flux in the mice with small tumor burdens (Table 1).

Mice briefly fasted or briefly fasted-refed a 250-mg test meal, with pair-fed controls. No significant differences in plasma FFA pool sizes were observed between briefly fasted TB and pair-fed control mice; therefore, the data were combined to obtain a best estimate for the population of briefly fasted animals. Similarly, there was no difference between the fasted-refed control and TB mice, and their plasma FFA data were also combined in order to obtain a best estimate of the pool sizes for the fasted-refed groups. Refeeding caused a significant 63% decrease ($p \leq 0.001$) in the mean plasma FFA levels of the combined TB and pair-fed control groups (Table 1).

This decrease in the mean plasma FFA pool size was consistent with the observed decreases in fractional rates of plasma FFA (linoleic acid) oxidation to breath CO_2

TABLE 1

Plasma FFA Pool Sizes, Fractional Rate Constants, and Replacement and Oxidation Rates in Lymphoma-Bearing and Control Mice Under Different Dietary Conditions[a]

Group	Pool sizes[b,c]	Fraction rate constants	Oxidation rates
Control			
Fed *ad libitum*	8.8 ± 0.43 (12[d]	0.20 ± 0.0086 (9)[d]	1.8 ± 0.13 (9)[d]
Briefly fasted	13 ± 0.49 (7)	1.1 ± 0.063 (5)	14 ± 0.90 (5)
Fasted-refed	4.8 ± 0.96 (9)	0.66 ± 0.050 (5)	3.2 ± 0.67 (5)
Tumor-bearing[e]			
Fed *ad libitum*	19 ± 1.6 (19)	0.84 ± 0.045 (8)	16 ± 1.6 (8)
Briefly fasted	13 ± 0.49 (7)	1.1 ± 0.057 (5)	14 ± 0.90 (5)
Fasted-refed	4.8 ± 0.96 (9)	0.75 ± 0.064 (5)	3.6 ± 0.79 (5)

[a] Plasma FFA pool sizes, μg-atoms FA-carbon/30 g body weight ± SE; fractional rate constants, per min ± SE; oxidation rates, μg-atoms FA-carbon/min/30 g body weight ± SE.

[b] Plasma FFA pool sizes, fractional rate constants, and oxidation rates correspond to M(1), L(10,1) and R(10,1), respectively, using the model and CONSAM analysis described earlier (5,11).

[c] Pool sizes of the *ad libitum*-fed groups have been published previously (5) and are taken from a separate group of animals adapted to the same conditions as those used for the tracer studies. Values for the briefly fasted control and TB groups did not differ significantly, as was also true for the briefly fasted-refed control and TB groups; therefore, the mean ± SE of both control and TB groups combined (*i.e.*, the combined fasted and the combined fasted-refed groups, respectively) are shown.

[d] Statistical analyses: pool sizes of control *vs.* TB groups, both fed *ad libitum*, were significantly different ($p \leq 0.001$); those of control and TB groups combined (the value shown in each case), fasted *vs.* fasted-refed, were also significantly different ($p \leq 0.001$); fractional rate constants of control *vs.* TB groups, both fed *ad libitum*, were significantly different ($p \leq 0.05$), as were those of control, fasted *vs.* fasted-refed groups ($p \leq 0.05$), and of the TB, fasted *vs.* fasted-refed groups ($p \leq 0.05$); oxidation rates (assumed equal to net plasma FFA transport rates) of control *vs.* TB groups, both fed *ad libitum*, were significantly different ($p \leq 0.05$), as were those of controls, briefly fasted *vs.* fasted-refed groups ($p \leq 0.05$), and of TB, briefly fasted *vs.* fasted-refed groups ($p \leq 0.05$).

[e] Small tumor burden group.

induced by feeding the small test meal to both control and tumor-bearing mice (Table 1). The fractional rates were reduced an average of 40% in controls ($p \leq 0.01$) and 32% in TB mice ($p \leq 0.01$). The fractional FFA oxidation rates did not differ in the fasted TB *vs.* fasted control groups, nor in the fasted-refed TB *vs.* fasted-refed control groups. This was also true for the oxidation rate (mass/time), where the oxidation rates fell from 14 units in both TB and control groups in the briefly fasted state to about 3–3.5 units in the fasted-refed state (Table 1). Thus, the net flux of FFA following the ingestion of a single 250-mg test meal decreased by 77% in both TB and pair-fed control mice ($p \leq 0.001$, each group, fasted *vs.* fasted-refed just 30 min after the mice began eating.

DISCUSSION

Lymphoma-bearing mice are thought to have a potent circulating lipid mobilizing factor (LMF) that causes them to lose their body fat (1). Initially, this was demonstrated using an essential FA, linoleate, to label the adipose TGFA of normal rats and by showing, indirectly, that linoleate was rapidly mobilized from these fat pads when plasma from lymphoma-bearing mice was injected into the normal mice (1). Therefore, one might expect lymphoma-bearing mice, who produce this LMF, to mobilize linoleate from their own adipose TGFA stores at an increased rate. However, no one has actually measured the rates of free linoleic acid transport and oxidation in lymphoma-bearing mice to confirm this hypothesis.

Our experiments indicate that, in lymphoma-bearing mice, linoleate is mobilized from adipose tissue and the free linoleic acid oxidized to CO_2 at the same rate as in controls if the mice are briefly fasted. This confirms earlier observations using a non-essential FA, palmitate (4,5). Therefore, if an active, circulating LMF is produced by this tumor (1), it does not appear to influence rates of adipose TGFA mobilization ("lipolysis") in the briefly fasted state.

However, FFA (free linoleic acid) transport and oxidation rates in lymphoma-bearing mice were greatly elevated over the control rates in the *ad libitum*-fed state. This had been shown to be the case for free palmitic acid in earlier studies with similarly maintained lymphoma-bearing mice (5). Although superficially these data indicated that ingestion of food failed to inhibit adipose tissue TGFA-linoleate mobilization in the tumor-bearing mice, additional data and experiments carried out in the present study strongly suggest that this was not the case. The appetites of the tumor-bearing mice were seriously impaired. Therefore, when fed *ad libitum* they apparently did not ingest enough nutrient to activate the mechanisms that normally inhibit adipose tissue TGFA lipolysis. However, after ingesting a small known test meal, previously fasted lymphoma-bearing mice responded promptly by inhibiting linoleate transport (from adipose tissue TGFA) to the same extent as did the controls.

This finding is consistent with our earlier studies of Ehrlich ascites tumor-bearing mice (15), but contrasts with those of Waterhouse and Kemperman (16), who studied human cancer subjects. The latter were shown to have a pronounced regulatory defect in their ability to suppress plasma FFA transport and oxidation when given many repeated small test meals of glucose (16,17).

Although decreased caloric food intake at night would account for body fat loss in anorexic tumor-bearing mice, additional direct and indirect effects of tumor- and macrophage-derived cytokines upon adipose tissue (18) cannot be ruled out. However, the physiological actions of cytokines would be difficult to demonstrate in anorexic animals. Other, potentially more suitable models to study cytokine action have been described in which TB rodents lose much of their body fat despite apparently normal caloric intakes (*e.g.*, refs. 19,20).

According to the hypothesis of Sauer and Dauchy (3), the rate of tumor growth (mitosis) is directly proportional to the rate of free linoleic acid transport to the tumor. Our data show that lymphoma-bearing mice are anorexic and have an abnormally rapid rate of free linoleic acid transport at night. Undoubtedly they supply linoleic acid and other essential FFA to their tumors much faster than would be the case if the animals were eating normally. However, as we have shown here, these TB animals clearly do not lack the metabolic regulatory processes that inhibit linoleate transport. Hypothetically, it should be possible to diminish cachexia, inhibit the rate of tumor growth, and thereby increase longevity of lymphoma-bearing mice, despite the reported existence of a circulating lipid mobilizing factor (1). This might be accomplished either by appropriate parenteral feeding or by overcoming their anorexia, *e.g.*, by blocking the inhibitory effects of TNF/cachectin (21) on appetite centers (22,23).

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Urinary Response to *in Vivo* Lipid Peroxidation Induced by Vitamin E Deficiency

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Experiments were carried out to measure the urinary excretion of free and conjugated malonaldehyde (MDA) and other thiobarbituric acid reactive substances (TBARS) in vitamin E deficient and vitamin E supplemented rats. From both dietary groups, six TBA positive fractions were isolated, in addition to that containing free MDA, by high-performance liquid chromatography (HPLC) on a TSK-GEL G-1000PW column. Three of the fractions isolated were found to be significantly increased in vitamin E deficiency. After acid hydrolysis, only one of the above compounds produced free MDA which indicated the presence of derivatized MDA. Only this fraction exhibited fluorescence at excitation 370 nm and emission 450 nm. The five other fractions formed 2,4-dinitrophenylhydrazones (2,4-DNPH), indicating the presence of carbonyl groups, but the derivatized MDA fraction did not. No significant differences were found in free MDA levels between the vitamin E deficient and the vitamin E supplemented groups.
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Lipid peroxidation is a degradative, free radical mediated process that primarily involves polyunsaturated fatty acids of biomembranes (1,2). The nonenzymatic autoxidation of polyunsaturated fatty acids is known to be accompanied by the formation of a complex mixture of products including aldehydes such as alkanals, alk-2-enals, 4-hydroxyalkenals, and malonaldehyde (3-5). Malonaldehyde (MDA), one of the most studied secondary products of lipid peroxidation, has been known to be of biological significance due to its reactivity with biomolecules including amino acids (6-8), proteins (9-14), and nucleic acids (15,16). Peroxidizing tissue samples produce significant amounts of thiobarbituric acid-reactive substances (TBARS). Several reports have indicated that a positive relationship exists between *in vivo* lipid peroxidation and urinary MDA levels. Draper *et al.* (17) showed, using high-performance liquid chromatography (HPLC) of the MDA/TBA complex, that urinary MDA excretion increased in response to vitamin E deficiency after administration of iron nitrilotriacetate. Gavino *et al.* (18) reported that a cod liver oil diet and iron overload induced increased excretion of TBARS in the urine of rats. Ekström *et al.* (19) demonstrated increased MDA (as measured by HPLC of its dinitrophenylhydrazone) excretion in urine from non-starved animals exposed to chloroform and found that there is good agreement between ethane exhalation and urinary MDA levels. A comprehensive review on malonaldehyde and TBA reactivity has recently appeared (20).

The present study was designed to separate, by HPLC, free MDA from other TBA test-positive materials of urine, to see whether other fractions were associated with vitamin E deficiency, and to determine whether they contained bound MDA.

MATERIALS AND METHODS

Animals and diets. Two groups of 10 Sprague-Dawley female, weanling rats were fed either a modified basal vitamin E-deficient diet developed by Draper *et al.* (21) adequate in all respects except for vitamin E (−E group) or a basal diet supplemented with 30 mg/kg of RRR-α-tocopheryl acetate (1.36 IU/mg, Sigma Chemical Co., St. Louis, MO) (NE group) for 38 wk. Diets and water were provided *ad libitum*. Sodium salt of sulfamerazine (Sigma) was added to drinking water (1 g/3.8 L) to prevent pulmonary infections.

Urine collection. At the 38th week of the experimental diets, the animals were individually held in stainless steel metabolic cages for 36 hr and had access to water *ad libitum*, but were deprived of food. To avoid direct effects of diet, the urine samples were collected during the final 24 hr into 50-mL graduated centrifuge tubes attached to urine collection funnels with screens to prevent contamination from feces. The tubes contained a few drops of toluene as a preservative. Urine volumes were accurately measured, and the samples were centrifuged to remove hair and stored at −70°C until analysis.

Thiobarbituric acid test of urine fractions. The levels of TBARS in urine samples were measured by the modified filtration procedure of Tarladgis *et al.* (22). Duplicate samples of 0.5 mL urine were mixed thoroughly with 3 mL of 5% trichloroacetic acid (TCA) and 1 mL of 0.06M TBA solution in screw-capped culture tubes. The mixtures were heated in an 80°C water bath for 90 min, cooled to room temperature, and centrifuged at 1360 × *g* for 15 min to remove a fine precipitate. The absorbance of the supernatant was read at 535 nm using a Beckman (Fullerton, CA) Model DU-8 spectrophotometer. MDA standards were freshly prepared from tetramethoxypropane (TMP) and treated in the same way as the test samples. The amounts of TBARS in urine were expressed as equivalents of MDA.

HPLC separation and free MDA determination of urine samples. Chromatographic separation and free-MDA analysis of urine samples were carried out by the HPLC method developed by Csallany *et al.* (23). Aliquots (2 to 3 mL) of urine were filtered through an Amicon cell equipped with a UMO5 ultramembrane (Amicon Corp., Danvers, MA) under 35 psi of nitrogen pressure. Fifty to 100 μL of clear filtrates were injected onto the HPLC system, which consisted of an Altex Model 110A solvent metering pump (Beckman Instruments, Berkeley, CA), Altex Model 210 sample injector (Beckman Instruments), Spectra-Physics Model SP-8400 variable wavelength detector (Spectra-Physics, Arlington, IL), and a Model SP-4100 computing integrator (Spectra-Physics).

HPLC separations were performed on a 7.5 mm i.d. ×

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Abbreviations: −E, vitamin E deficient group; HPLC, high-performance liquid chromatography; NE group, 30 mg/kg of RRR-α-tocopherol acetate group; MDA, malonaldehyde; TBARS, thiobarbituric acid reactive substances; TMP, tetramethoxypropane; 2,4-DNPH, 2,4-dinitrophenylhydrazone.

30 cm Spherogel-TSK 1000 PW size exclusion [excluded volume 4.7 mL, total vol 9.7 mL, exclusion limit 1000 MW column (Beckman Instruments)] eluted with a mobile phase of 0.1 M phosphate buffer, pH 8.0, at a flow rate of 0.6 mL/min. Absorbance was monitored at 267 nm. Seven to eight urine samples from each dietary group were analyzed in duplicate measurements. The amount of free MDA was measured from peak area response, determined from a standard curve prepared with authentic MDA. The lowest amount of MDA that can be determined by this HPLC technique is approximately 1 ng per injection (23). The relationship between the other HPLC separated, urinary fractions and the dietary treatments was examined by comparing the mean peak areas (expressed as peak area/day/100 g body weight) of each urinary fraction between the two dietary groups.

Isolation of the individual urine fractions. Fractions were isolated from urine by pooling corresponding fractions from three 200 μL injections of rat urine. The pooled fractions were diluted uniformly to 5 mL. Pooled fractions were checked for homogeneity by re-injecting a 200-μL aliquot on the same HPLC system, and 4.5 mL of each fraction was lyophilized in a Virtis freeze dryer (Virtis Res. Equipment, Gardiner, NY).

TBA reactivity of the isolated fractions. Residues from 16 fractions were dissolved in 2 mL of deionized water and 1 mL aliquots (equivalent to 0.27 mL urine) were subjected to the TBA test procedure, described previously. The TBA chromophores in each urine fraction and that of standard MDA were extracted with 2 mL of n-butanol. Absorption spectra of the butanol extracts were measured over the wavelength range of 450–600 nm.

Absorption spectra of the "TBA test-positive" urine fractions. Lyophilized fractions (that had been shown previously to be "TBA test-positive") were dissolved in 2 mL deionized water. The absorption spectrum of each fraction was obtained against a blank prepared by the same procedure using a Beckman DU-8 spectrophotometer equipped with a wavelength scan compuset.

Fluorescence measurement of the "TBA test-positive" urine fractions. Lyophilized residues of "TBA test-positive" fractions were dissolved in 1.5 mL of HPLC grade water and filtered through a 0.45 μm Magna nylon 66 membrane filter (Micron Separation Inc., Honeoye Falls, NY). The filtrates (200 μL) were injected onto a Spherogel-TSK 1000 PW column, and fluorescence was determined with a Perkin-Elmer model 650-105 fluorescence spectrophotometer (Perkin-Elmer Co., Norwalk, CT) at 370 nm excitation and 450 nm emission, and 405 nm excitation and 465 emission.

Acidic hydrolysis of the fluorescent fraction. A lyophilized sample of the fluorescent fraction (retention time 19 min) was dissolved in 0.5 mL HPLC grade water, and then 1 mL of 10% trichloroacetic acid was added to bring the pH to 2.0. The acidic mixture was heated in a 80°C water bath for 90 min, cooled to room temperature, neutralized to pH 8.0 with 30 μL of saturated NaOH solution, and filtered through a Magna nylon 66 membrane filter. The filtrate of the hydrolyzed sample was analyzed for free MDA by the HPLC method described previously. The identity of the free MDA peak was confirmed by cochromatography of a mixture of the hydrolysate with authentic MDA.

Formation of 2,4-dinitrophenylhydrazones of "TBA test-positive" urine fractions. Lyophilized aliquots of the six "TBA test-positive" urine fractions were dissolved in 2 mL of HPLC grade water, and the 2,4-dinitrophenylhydrazones (DNPH) were prepared by the method described by Esterbauer *et al.* (24). Hexanal (Sigma), *trans*-2-heptenal (Aldrich Chemical Co., Milwaukee, WI), and acetone (Fisher Scientific, Fairlawn, NJ) were used as standards representative of an alkanal, 2-alkenal, and alkanone, respectively.

RESULTS AND DISCUSSION

In the present experiments vitamin E deficient rats excreted significantly higher levels of total TBARS in urine, calculated on the basis of 24-hr urine volume and rat body weight (BW), compared to vitamin E-supplemented rats (Fig. 1). This demonstrates that total urinary TBARS levels respond to the increased *in vivo* lipid peroxidation associated with vitamin E deficiency. However, the actual amount of urinary free MDA in both groups was much less than the total TBARS when expressed as equivalents of MDA. Furthermore, in spite of a large difference in total urinary TBARS levels between the vitamin E-deficient group and the normal group, the difference in free MDA levels in these two groups was not statistically significant (Fig. 1). These observations indicate that the amount of free MDA in the urine alone does not reflect *in vivo* lipid peroxidation. Although some investigators (16) have reported increased urinary MDA in the vitamin E-deficient rat, their results may not represent only free MDA present in the urine because of their assay method is based on the final measurement of the TBA derivative of MDA (only) by HPLC. MDA may be released from its derivatized forms by acid hydrolysis during analysis. We have reported previously that free MDA levels in liver tissue were 15 times higher in animals fed a vitamin E-deficient diet than in control animals (14). However, differences in urinary free MDA levels were not observed between the vitamin E-deficient animals and the control group in the present experiments. A possible explanation could be that most MDA is metabolized by the liver (25) and thus only a minor fraction of the available free MDA

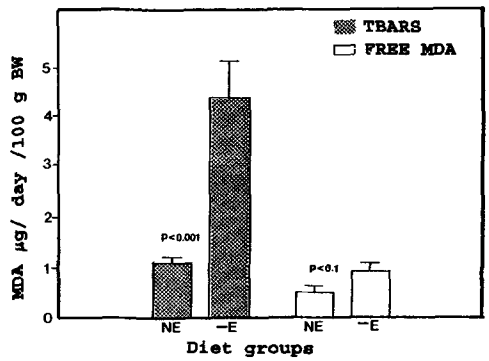

FIG. 1. Comparison of total urinary TBA-reactive substances (TBARS) and free malonaldehyde (MDA) levels in 38-wk old normal and vitamin E-deficient rats. Values represent the mean ± SEM of 10 animals per dietary group.

is excreted in the urine. Another possibility is that the reactivity of free MDA could lead to its combination with other compounds and to excretion in a derivatized form in the urine (26).

A typical HPLC chromatogram of a rat urine sample, separated by Spherogel-TSK 1000 PW column at 267 nm, is shown in Figure 2. Free MDA is well separated (R_t 44 min) from other compounds and this system has the additional advantage of allowing the fractionation of urine components according to molecular size as well. The retardation of MDA well beyond the total volume of the size exclusion column indicates that additional separation modes are available with this column. Other investigators have used anion exchange chromatography to fractionate the MDA-containing components of rat urine, and found that most (75%) of the MDA was present as *N*-α-acetyl-ε-(2-propenal)lysine (10).

The peak areas of the 13 major urine fractions were calculated on the basis of 24-hr urine volume and rat body weight (BW) to determine which of the urinary fractions responded to vitamin E deficiency (Fig. 3). Mean values were compared between vitamin E-deficient and control groups. The areas of six of these fractions (R_t 19,21,29,48,56, and 62) were larger in the vitamin E-deficient group than in the control group. This may indicate that the six urine fractions are vitamin E-deficiency dependent, and therefore may be related to *in vivo* lipid peroxidation.

Each HPLC separated and purified urine fraction was reacted with TBA reagent. Positive TBA test results among the sixteen purified individual urine fractions are shown in Table 1. Six fractions, in addition to the isolated MDA fraction (R_t 44), were found to be "TBA test-positive" regardless of the dietary treatments. The absorption spectra of TBA chromophores of these six fractions showed significant peaks near that of standard MDA. Accordingly, the level of total urinary TBARS reflects contributions at 533 nm of all the TBA chromophores of these fractions. Three of the "TBA test-positive" fractions (R_t 19,21, and 29) were significantly higher in vitamin E deficiency. The three fractions, therefore, appear to be responsible for the increased levels of TBARS in the urine of rats under these dietary conditions. *N*-α-acetyl-ε-(2-propenal)lysine has been reported (10,27) to be the major contributor to the TBARS of urine. This compound, as well as MDA conjugates of other amino acids, coelutes with the 19-min fraction found in the present study. The amount of this and other amino acid conjugates of MDA in fraction R_t 19, as well as the identity of the compounds in the other fractions, is currently under investigation. Absorption maxima of "TBA test-positive" urine fractions are shown in Table 1. All of these fractions showed characteristic absorption maxima in the ultraviolet (UV) range of 230–270 nm. Fraction R_t 44 (Table 1) was confirmed as MDA from its absorption maximum at 266 nm, which corresponds to that (267 nm) of standard MDA at neutral or alkaline pH. Three of the fractions (R_t 19,21, and 29), which are both "TBA test-positive" and are significantly increased in vitamin E deficiency, have their maximum absorptions at 230, 260, and 259 nm, respectively. Condensation products of MDA and primary amino groups of biomolecules have been known to possess fluorescence due to their Schiff's base type structure (8,28). The existence of Schiff's bases

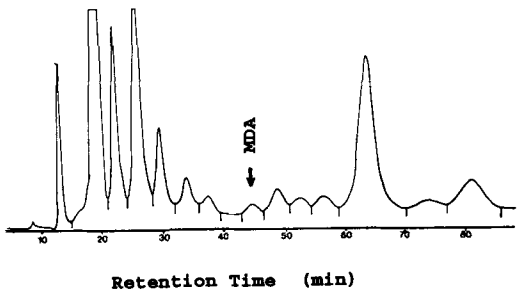

FIG. 2. Typical HPLC chromatogram of a rat urine sample. Separation conditions: Spherogel-TSK 1000PW size exclusion column; mobile phase, 0.1 M sodium phosphate buffer, pH 8.0; flow rate, 0.6 mL/min; monitoring wavelength 267 nm; ambient temperature.

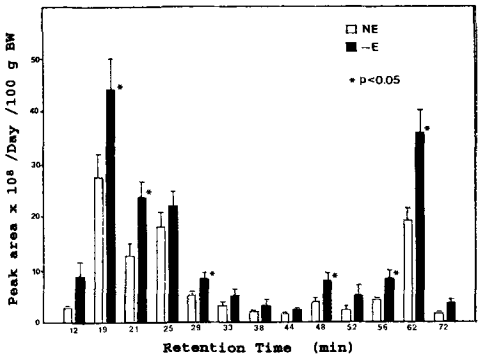

FIG. 3. Comparisons of HPLC separated urine fractions of rats fed a vitamin E-deficient (−E) or normal (NE) diet for 38 wk. Values represent the mean ± SEM of 7 samples/group; *p<0.05 by Student's t-test.

in these "TBA test-positive urinary fractions was examined by measuring their fluorescence characteristics. Excitation and emission wavelengths at 370 nm/450 nm and 405 nm/465 nm were selected for the measurements of Schiff's bases derived from MDA and amino acids (8) and of Schiff's bases formed from MDA and albumin or other proteins (29), respectively. Only one fraction (R_t 19) was found to possess fluorescence at Ex 370 nm/Em 450 nm, indicating the presence of a Schiff's base (Table 2). The fluorescent urine fraction (R_t 19) was hydrolyzed under acidic conditions (pH 2.0) to confirm this possibility and the existence of MDA in a Schiff's base form. The HPLC chromatograms of this fraction prior to and after acid hydrolysis, cochromatography of MDA standard with the hydrolyzed fraction 19, and standard MDA are shown in Figure 4. Acidic hydrolysis of the fluorescent fraction (R_t 19) resulted in the release of free MDA. The presence of free MDA was confirmed by cochromatography of the hydrolysate with standard MDA. This result indicates that the fluorescent fraction (R_t 19) contains MDA as an acid-hydrolyzable Schiff's base-type derivative. The other "TBA test-positive" fractions were also hydrolyzed under the same conditions, but none of these fractions released free MDA after hydrolysis. Each fraction was examined

TABLE 1

Characteristics of TBA Chromophores of Urine Fractions

Urine fractions retention times (min)	Absorption maxima (nm)					
	TBA chromophores in n-butanol extract[a]	Isolated fractions			DNPH[b,c]	
12	531.70	265	212		350.03	256.37
19	531.37	230	213		ND[d]	
21	533.37	260	270		368.40	
25	533.35	254	215		369.20	254.70
29	534.18	259	207		368.38	257.03
33	533.37	261	234	208	370.05	257.03
44	530.88	266	209		ND	

[a]MDA R_t 44 min; λ max = 530.88.

[b]DNPHY, dinitrophenylhydrazone.

[c]Standards: hexanal DNPH, 362.53; hept-2-enal DNPH, 378.37, 285.53; acetone-DNPH, 367.53, 275.00.

[d]ND, not detected.

TABLE 2

Fluorescence Measurement of the "TBA Test-Positive" Urine Fractions

Urine fractions retention times (min)	Excitation 370/emission 450 (nm)	Excitation 405/emission 465 (nm)
12	ND[a]	ND
19	Fluorescent	ND
21	ND	ND
25	ND	ND
29	ND	ND
33	ND	ND

[a]ND, not detected.

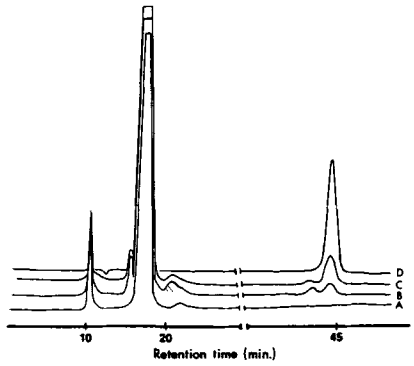

FIG. 4. HPLC chromatograms of urine fraction 19. Chromatogram A, before hydrolysis; B, after acid hydrolysis; C, cochromatogram of MDA standard and hydrolyzed fraction 19; D, standard MDA. HPLC separation conditions: Spherogel-TSK 1000 PW column; mobile phase, 0.1 M sodium phosphate buffer, pH 8.0; flow rate, 0.6 mL/min; monitoring wavelength, 267 nm.

for the presence of MDA using the method of Bird *et al.* (30), whereby the TBA/MDA (2:1) complex is isolated by HPLC. Fraction (R_t 19) was the only urine fraction containing MDA in a derivatized form.

A variety of secondary carbonyl products such as alkanals, alk-2-enals, 4-hydroxyalkenals, MDA, and alkanones are produced by peroxidation of unsaturated fatty acids (5), and several of these products have been reported to react with the TBA reagent (31,32). Kosugi *et al.* (33) have studied the reaction of alk-2-enals with TBA and found that under certain conditions red pigments were produced which were indistinguishable spectrophotometrically from the 2:1 complex of TBA with MDA. These compounds also exhibited similar retention times during reverse-phase HPLC. The molar absorptivity of the TBA complex with MDA was much greater than that of the complexes with other aldehydes, however. Kosugi *et al.* (34) have more recently examined the products of the TBA reaction with various peroxidized lipids and concluded that the yield of the TBA/MDA (2:1) product, as measured by absorbance, fluorescent intensity and HPLC, was much greater than the MDA contents of the preparation as measured by direct chemical analysis. Thus, some products of lipid peroxidation may produce MDA during the measurement of the TBA reaction. As described previously, five of the "TBA test-positive" urine fractions obtained in this study contained no MDA in a derivatized form. The reactivities of these non-fluorescent fractions with the TBA reagent may have been due to the presence of carbonyl compounds other than MDA. The presence of carbonyl groups was tested by the formation of 2,4-dinitrophenylhydrazones. The absorption maxima of the derivatives of urine fractions and standard carbonyl compounds are listed in Table 2. The fluorescent fraction (R_t 19) was the only fraction that did not form a 2,4-DNPH. It should be noted that free MDA, a bifunctional aldehyde, yields a pyrazole derivative instead of a hydrazone when reacted with dinitrophenylhydrazine (35). The major absorption maxima of the derivatives of the four fractions (R_t 21,15,29,and 33) are quite similar to that of a derivative of acetone which may indicate the presence of keto groups in these fractions. The carbonyl groups present in the non-fluorescent urine fractions may be derived from short- or medium-chain secondary lipid peroxidation products and seem to be responsible for the reactivities of these fractions with the TBA reagent. Fur-

ther investigations are needed to reveal the identity of the vitamin E dependent, urinary TBA reactive carbonyl-containing compounds.

Although MDA may be an important product of lipid peroxidation *in vitro*, subsequent metabolism of this compound may complicate the use of free MDA excretion as a measure of lipid peroxidation *in vivo*. Others have found conjugated MDA in the urine to be increased in vitamin E deficiency (17) but this increase is superimposed upon the considerable background of conjugated MDA acquired from the diet. The present finding of substances which do not release MDA upon hydrolysis but are profoundly increased during vitamin E deficiency may provide more satisfactory measures of *in vivo* lipid peroxidation.

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Separation and Spectral Data of the Six Isomeric Ozonides from Methyl Oleate

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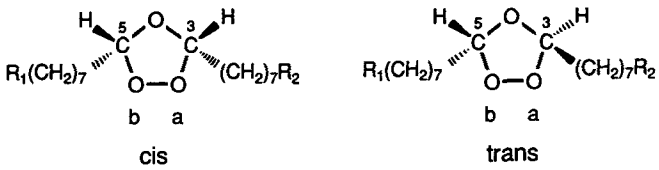
The structures of the products obtained on ozonation of methyl oleate have been re-examined. The assignments for the six isomeric ozonides of methyl oleate have been made by ^1H and ^{13}C nuclear magnetic resonance (NMR), which were consistent with the retention times observed in high-performance liquid chromatography; the assignments were confirmed by mass and infrared spectroscopy. Two triplets for the ozonide ring protons of the *cis* and *trans* isomers in the normal (MOO1) and the two cross ozonides (MOO1 and MOO3) can be resolved by 400 MHz NMR. For MOO1 and MOO3, where the two ring carbons are equivalent, two peaks for the ring carbons of each cross ozonides are resolved in the ^{13}C NMR spectra, one for the *cis* and one for the *trans* isomer. For MOO2, four peaks for the ring carbons are resolved in the ^{13}C NMR spectra, two for the *cis* and two for the *trans* isomer. *Lipids 27,* 129–135 (1992).

The Criegee mechanism for the reaction of ozone with alkenes in solution has been thoroughly investigated (1). The detection of cross ozonides and *cis* and *trans* isomers has been taken as evidence for the formation of carbonyl oxides as intermediates (1). Both *cis* and *trans* isomers of the normal ozonide, methyl 5-octyl-1,2,4-trioxolane-3-(8′-octanoate) (MOO2), and two cross ozonides, 3,5-dioctyl-1,2,4-trioxolane (MOO1) and dimethyl 1,2,4-trioxolane-3,5-(8′,8′-dioctanoate) (MOO3), produced upon ozonation of methyl oleate were first isolated in the early 1960's by Riezebos *et al.* (2) and Privett and Nickell (3,4). Later, Lorenz and Parks (5) identified similar isomers upon ozonation of smaller asymmetric alkenes. Subsequently, several papers (6–11) were published describing the cross ozonide yields and *cis* and *trans* ratios. Since the 1970's, a number of studies also have been concerned with the biological effects of fatty acid ozonides (12–21).

Lung surfactant and cell membrane lipids may be important targets for inhaled ozone (22–29), as oleate is one of the major unsaturated fatty acids in lung surfactants (30) and in cell membrane lipids (31). Hence, oleate ozonides may be formed upon inhalation of ozone-containing air.

The *cis* and *trans* isomers of the normal and cross ozonides of methyl oleate have previously been characterized by thin-layer chromatography (TLC), infrared (IR), and ^1H nuclear magnetic resonance (NMR) (2–4). A recent report from our laboratory also provided some ^1H NMR and ^{13}C NMR data for MOO2 (20). Scheme 1 shows the structures (and abbreviations) of the six isomeric ozonides of methyl oleate. We report here reversed-phase high-performance liquid chromatography (HPLC) retention times, UV, and high resolution ^1H and ^{13}C NMR spectral data for all six isomeric ozonides of methyl oleate, along with mass and FT-IR (Fourier transform-IR) spectral data for the normal and cross ozonides of methyl oleate.



Name of ozonide	Abbreviation	R_1	R_2	M.W.
"Cross"	MOO1	CH_3	CH_3	300
"Normal"	MOO2	CH_3	$-COOCH_3$	344
"Cross"	MOO3	CH_3COO-	$-COOCH_3$	388

SCHEME 1. The six isomeric ozonides from methyl oleate.

EXPERIMENTAL PROCEDURES

Materials. Methyl oleate (>99%) was purchased from Sigma Chemical Co. (St. Louis, MO). Both analytical TLC plates (PE Sil G/UV, F-254, 250 μm layer) and preparative TLC plates (Silica Gel 60A, F-254, 20 × 20 cm, 1000 μm layer) were purchased from Whatman International, Ltd. (Maidstone, England).

Preparation of the ozonation mixture from methyl oleate. Using a procedure modified from that described by Ewing *et al.* (20), 4.5 g of methyl oleate was dissolved in 80 mL of dry pentane in a 200-mL, three-neck flask connected to a trap containing a 10% KI sollution. The reaction vessel was then chilled in an ice-water bath. An Orec ozonator (Ozone Research & Equipment Corp., Phoenix, AR; Model 03V10-0) was used to generate a 1.2% (v/v) ozone-in-oxygen gas stream, which was bubbled at 200 mL/min through the chilled, continuously stirred reaction solution. Pentane was added to keep the volume of the reaction solution constant during ozonation. The reaction was stopped after about 3 hr when the KI solution in the trap became yellow. At the end of the reaction, the absence of methyl oleate was determined by TLC. Unreacted ozone was removed by slowly bubbling nitrogen through the reaction solution. The solvent was removed using a rotary evaporator under vacuum at room temperature, leaving about 4.8 g of an oily material with a unique smell.

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Abbreviations: CI, chemical ionization; FT-IR, Fourier transform infrared; HPLC, high-performance liquid chromatography; IR, infrared; MOO1, one of the cross ozonides from methyl oleate (3,5-dioctyl-1,2,4-trioxolane); MOO2, the normal ozonide from methyl oleate (methyl 5-octyl-1,2,4-trioxolane-3-(8′octanoate)); MOO3, the second cross ozonide from methyl oleate (dimethyl 1,2,4-trioxolane-3,5-(8′,8′-dioctanoate)); MOO1, MOO2, and MOO3 refer to both *cis* and *trans* isomers (see Scheme 1 for structures of the six isomeric ozonides); NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

Analysis of the ozonation mixture. A Whatman analytical TLC plate was developed using a mobile phase of diethyl ether/petroleum hydrocarbon (1:9, v/v, bp 30–60°C). A Hewlett-Packard (Palo Alto, CA) chromatograph (Model HP 1090 LC chromatograph) with a reversed-phase Hewlett-Packard ODS Hypersil, 5 μm, 200 × 2.1 mm column was used. The mobile phase was 50% methanol/water, increased linearly to 100% methanol over 15 min, and then was held at 100% methanol for 10 min. The flow rate was 0.45 mL/min and the eluent was continuously monitored at 210 nm. Monitoring at 230 nm gave similar results.

Isolation of the cis/trans *pairs of the normal and cross ozonides.* The oily material from ozonation was separated into one pair of normal and two pairs of cross ozonides using fluorescence Whatman preparative TLC plates. The preparative TLC plate was developed using diethyl ether/petroleum hydrocarbon (5:95, v/v, bp 30–60°C).

Isolation of the cis *and* trans *normal ozonides.* A reversed-phase Hewlett-Packard ODS Hypersil, 5 μm, 200 × 4.6 mm column was used. The mobile phase, 93% methanol/water, was used isocratically at a flow rate of 1.0 mL/min.

Analysis of the ozonide yields from ozonation of methyl oleate in hexane. About 10 mg of methyl oleate was dissolved in 2.4 mL of hexane and ozonated for 2 min using 1.2% ozone-in-oxygen. The solvent was evaporated, methanol was added to give a total volume of 2.4 mL, and the solution was directly subjected to HPLC analysis. The ozonide yields were calculated using the external standard method (21) and the ratios of the *trans* to *cis* isomers were calculated using the ratios of corresponding HPLC peak areas.

Spectrometry. UV spectra were determined on a Hewlett-Packard HPLC instrument equipped with a diode array detector. For chemical ionization (CI) mass spectra, a unit-resolution Finnigan MAT mass spectrometer (model Finnigan TSQ 4500) was used with either methane or isobutane at 0.5 torr. The direct probe mode was used, and the source temperature was set at 50°C. For Fourier transform IR (FT-IR), a film was deposited on an NaCl disk, and spectra were obtained on a Perkin-Elmer FT-IR 1700X instrument with a 1 cm^{-1} resolution. ^1H NMR spectra were obtained at 400 MHz on a Bruker AM-400 NMR spectrometer equipped with an Aspect 3000 computer or at 200 MHz on a Bruker AC-200 NMR spectrometer; both instruments were also used for recording ^{13}C NMR spectra. The solvent was $CDCl_3$ for both ^1H and ^{13}C spectra. A trace amount of $CHCl_3$ in $CDCl_3$ served as a reference, giving rise to signals at 7.26 ppm in ^1H and at 77.00 ppm in ^{13}C NMR.

RESULTS AND DISCUSSION

Assignments of the normal and cross ozonides. Three types of methyl oleate ozonides can be identified by analytical TLC of the ozonation mixture (see Table 1); these correspond to one normal ozonide (MOO2) and two cross ozonides (MOO1 and MOO3), all of which consist of both *cis* and *trans* isomers (see Scheme 1 for structures). The assignments for the normal ozonide and cross ozonides isolated by preparative TLC are consistent with mass, IR, and NMR data. The mass spectral data provide the molecular weights of the protonated parent, $[M + H]^+$, for MOO1, MOO2, and MOO3. The IR data (see Table 2) show no carbonyl group for MOO1, one carbonyl for MOO2, and two carbonyls for MOO3 based on the absorption at *ca.* 1740 cm^{-1} (2).

TABLE 1

Normal TLC R_f Values and Reversed-Phase HPLC Retention Times (R_t), for Methyl Oleate d the Ozonides from Methyl Oleate

Spot or peak[a]	R_f[b]		R_t, min[c]
MMOO1	—	0.86	18.1 *(trans)* 17.9 *(cis)*
Methyl oleate	—	0.74	17.4
MOO2	0.53 *(trans)* 0.47 *(cis)*	0.51	16.3 *(trans)* 16.1 *(cis)*
MOO3	—	0.20	14.1 *(trans)* 13.9 *(cis)*

[a] See Scheme 1 for structures.

[b] R_f values in the first column are taken from ref. 35; R_f values in the second column are our data.

[c] Our data.

TABLE 2

IR Data for the Normal and Cross Methyl Oleate Ozonides and Methyl Oleate (in cm^{-1})

Ozonide[a]	The ring C-O bond[b]			Ester carbonyl[b]		
MOO1	1108	1110		N.A.[c]	N.A.	
MOO2	1109	1110	1109	1742	1730	1741
MOO3	1109	1110		1742 1734	1730	
Methyl oleate	N.A.			1744		

[a] See Scheme 1 for the structures.

[b] The first column lists our data; data in the second column are from ref. 2; data in the third column are from ref. 20.

[c] N.A., no absorptions.

The NMR data support the assignments of the normal and cross ozonides (Table 3). Neither ester methyl protons (*ca.* δ 3.66 ppm) nor ester carbonyl carbons (*ca.* δ 174 ppm) are detected in the NMR spectrum of MOO1, but there are two terminal methyl groups (δ 0.88 ppm, 6H) indicated in the ^1H spectrum and a peak at δ 14.05 ppm in the carbon spectrum. There is just one ester carbonyl in MOO2, as indicated by the absorption at δ 3.66 ppm (3H) and δ 174.2 ppm for the ester methyl protons and ester carbonyl carbon, respectively. Since δ 0.88 ppm (3H) and δ 14.06 ppm peaks corresponding to the terminal methyl protons and the terminal methyl carbon are detected for MOO2, one terminal methyl group is present in this ozonide. There are two ester methyls in MOO3 since δ 3.65 ppm (6H) and δ 174.2 ppm peaks are detected for the ester methyl protons and carbonyl carbons, but no terminal methyl since neither δ 0.88 ppm nor δ 14 ppm peaks are detected.

Assignment of the cis *and* trans *isomers.* Because the *cis* isomer has a larger dipole moment (32,33), the peak with shorter retention time in reversed-phase HPLC was assigned to the *cis* isomer and the peak with the longer

TABLE 3

^1H and ^{13}C NMR Data for Methyl Oleate and the Six Ozonides from Methyl Oleate (in ppm)

	MOO1[a]	MOO2[a]	MOO3[a]	Methyl oleate
Proton NMR				
Ozonide ring	(c)[b] 5.19 (t, 1H) (t)[c] 5.14 (t, 1H)	(c) 5.18 (t, 1H) (t) 5.13 (t, 1H)	(c) 5.17 (t, 1H) (t) 5.12 (t, 1H)	5.34[d] (m, 2H)
Ester methyl	—	(c) 3.66 (s, 3H) (t) 3.66 (s, 3H)	3.65 (s, 6H)	3.65 (s, 3H)
Terminal methyl	0.88 (t, 6H)	(c) 0.87 (t, 3H) (t) 0.88 (t, 3H)	—	0.87 (t, 3H)
Carbon NMR				
Ozonide ring	(t) 104.33 (c) 104.28	(t) 104.34 (C-5) (t) 104.23 (C-3) (c) 104.26 (C-5) (c) 104.18 (C-3)	(t) 104.24 (c) 104.18	129.99[d] 129.73[d]
Ester carbonyl	—	(t) 174.21 (c) 174.22	174.23	174.22
Terminal methyl	14.05	(t) 14.06 (c) 14.06	—	14.06

[a]See Scheme 1 for the structures. [b]*c: cis*-Isomer. [c]*t: trans*-Isomer. [d]From the double bond.

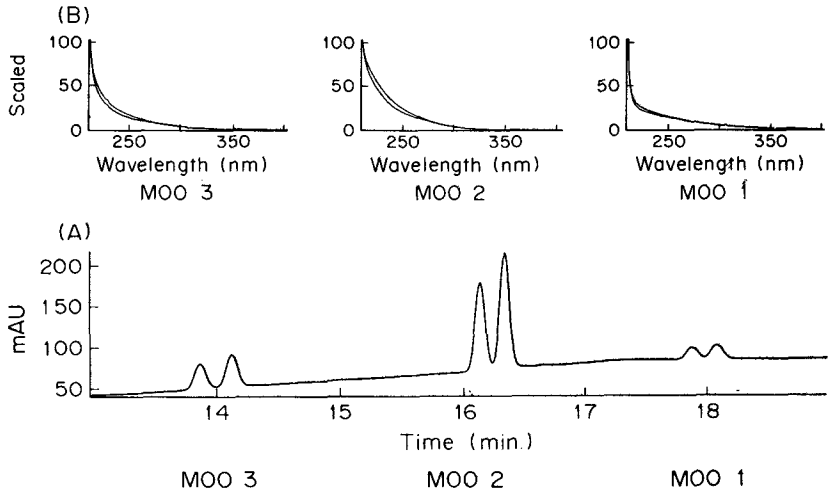


FIG. 1. A. Reversed-phase HPLC chromatogram for the six ozonides of methyl oleate. B. UV spectra for the six ozonides of methyl oleate. In the Figure, the *trans* isomer is always the top curve, whereas the *cis* isomer is always the bottom curve of the spectra.

retention time to the *trans* (see Table 1). This assignment is consistent with the literature (3,34,35), where the isomer with the smaller R_f value in normal-phase TLC is assigned the *cis* configuration and the isomer with the larger R_f value the *trans* form. The assignment was further confirmed by the faster rate of hydrolysis observed for the *cis* isomer (21), since the *cis* isomer has higher free energy and higher reactivity than the *trans*. Similarly, the *cis* ozonide was reported to undergo reduction faster than the *trans* form (36,37). Our ^1H NMR data support this assignment as well, since it is known that the ^1H resonance signal of the ring proton of the *cis* isomer occurs at lower field than that of the *trans* isomer (34).

Analytical yields determined by HPLC for the ozonides formed by ozonation of methyl oleate in hexane. Figure 1A shows a reversed-phase HPLC chromatogram for the six possible ozonides generated by ozonation of 0.013 M methyl oleate in hexane at 0°C under conditions where no methyl oleate is recovered. Each adjacent pair of peaks corresponds to a single TLC spot, in which the peak with the shorter retention time is assigned to the *cis* isomer.

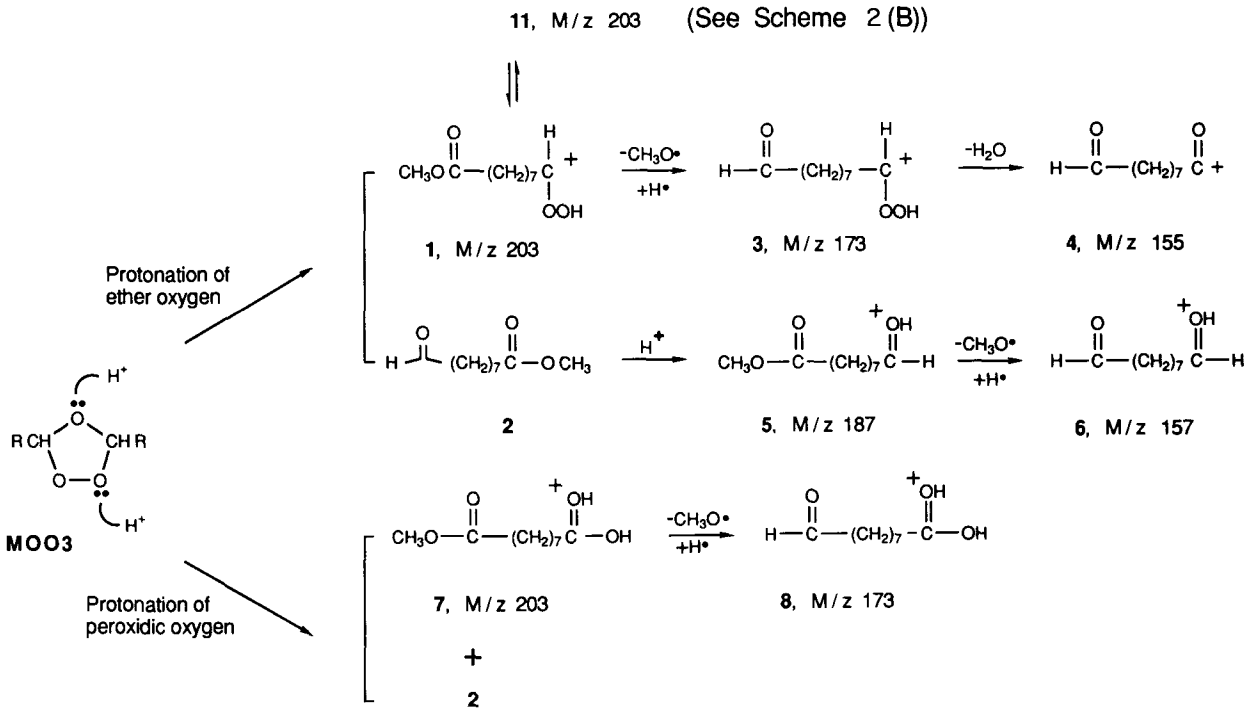
The total ozonide yield in Figure 1A is 89% as measured by HPLC based on an external standard (21). The yields of the individual compounds are 9.9%, 66%, and 13% for MOO1, MOO2, and MOO3, respectively, in relative yields of 0.15:1.0:0.19. Riezebos *et al.* (2) reported relative yields of 1:2:1 for MOO1, MOO2, and MOO3 generated by ozonation of 0.34 M methyl oleate in light petroleum at 10°C. However, when they ozonized 0.017 M methyl oleate at −65°C, they could not detect any cross ozonides (2).

Using a 0.013 M solution of methyl oleate in hexane at 0°C, we obtain a *trans* to *cis* ratio of 1.3 for MOO2, compared to 1.1 by ozonation of 0.014 M methyl oleate in pentane at −70°C (3). Both the cross ozonide yield and the *trans* to *cis* ratio of each ozonide are higher at a higher ozonation temperature because of the "solvent cage effect" and equilibration between *syn*- and *anti*-carbonyl oxides (21).

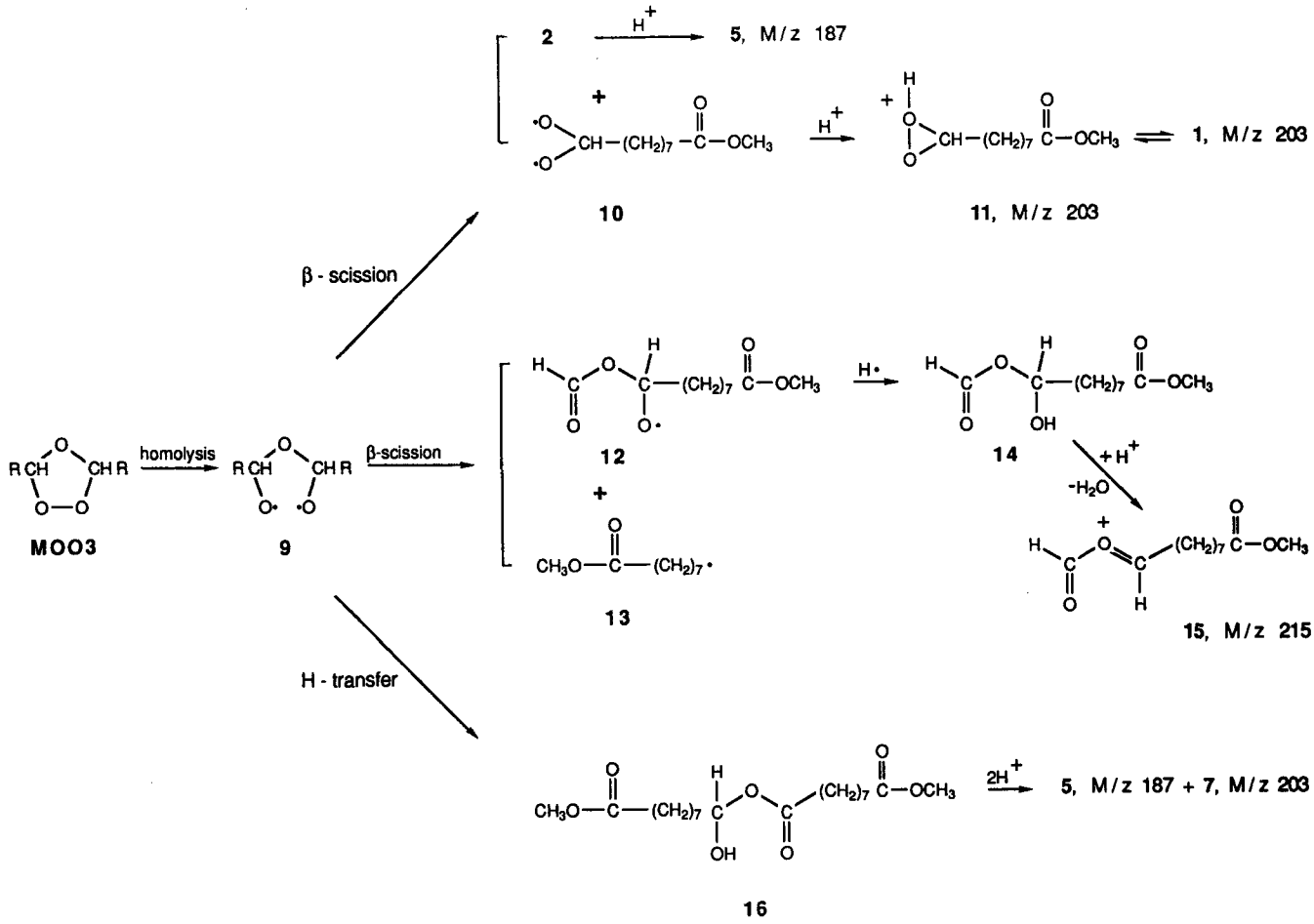
The UV spectra of the ozonides. The UV spectra of all six ozonides are shown above the HPLC chromatogram (Fig. 1B). No strong characteristic absorptions were detected; the absorption is strongest in the vicinity of 200 nm and rapidly decreases with increasing wavelength. The normal ozonides of methyl oleate have broader absorption curves than the cross ozonides. For either MOO1, MOO2, or MOO3, the *cis* and *trans* isomers have similar absorption curves. For better sensitivity and baseline stability, a wavelength of 210 nm was chosen for HPLC detection. [Analysis at 230 nm gave similar results (21).]

Mass spectra and fragmentation patterns. Fragmentation patterns are suggested for MOO3 by heterolytic (Scheme 2A) and homolytic (Scheme 2B) decompositions, based on the mechanisms of acidolysis (38) and thermal decomposition (20) of the ozonides, as well as the fragments generated in mass spectra (21). Scission of the ring C-O, peroxy O-O, and ester methoxy CO-OCH_3 bonds occur in either case. The fragmentation pattern for MOO1 is illustrated in Scheme 2C, where ions **17**, **18**, and **19** are generated in the same way as ions **11**, **2**, and **7** in Schemes 2A and 2B, respectively. The fragmentation pattern for MOO2 is similar to the patterns shown in Schemes 2A, 2B, and 2C. When methane was used as the CI gas, the MOO3 spectrum showed the protonated parent ion, $[M + H]^+$, at *m/z* 389 (15%); and the methane adducts, $[M + C_2H_5]^+$ and $[M + C_3H_5]^+$ at *m/z* 417 (6.2%) and 429 (1.5%). The peak at *m/z* 371 (6.2%) is formed by dehydration of the protonated parent, $[M + H - H_2O]^+$, and 357 (6.2%) is derived from the protonated parent having two oxygen atoms less, $[M + H - O_2]^+$. Other fragments can be found in Schemes 2A and 2B. By using methane as the CI gas, the mass spectrum of MOO1 provides peaks at *m/z* 301 (18%) for the protonated parent of $[M + H]^+$ and at *m/z* 329 (1.0%) for methane adducts of $[M + C_2H_5]^+$. The peak at *m/z* 285 (14%) results from the loss of one oxygen atom from the protonated parent, $[M + H - O]^+$. Other fragments can be found in Scheme 2C. Using isobutane as the CI gas, MOO2 shows the protonated parent ion, $[M + H]^+$, at *m/z* 345 (3.0%); and the isobutane adduct, $[M + C_4H_9]^+$, at *m/z* 401 (0.05%). The peak at *m/z* 373 is derived from the isobutane adduct upon loss of a carbonyl, $[M + C_4H_9 - CO]^+$; and 317 (1.5%) is derived from the protonated parent upon loss of a carbonyl, $[M + H - CO]^+$. Other fragments are the same as those from both MOO1 and MOO3. The peak at *m/z* 141 (50%) in the spectra of MOO1 is ion **21**, possibly arising from hydride abstraction from the C_9 aldehyde $[18 - H]^+$. The peak at *m/z* 159 (100%) is the base peak for MOO1, and 187 (100%) is the base peak for both MOO2 and MOO3.

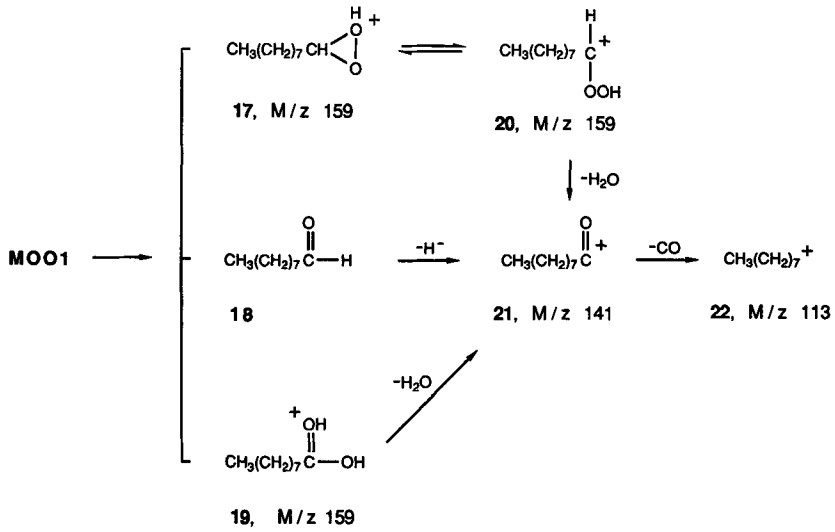
NMR studies on methyl oleate ozonides. As discussed earlier, the NMR peaks of the terminal methyl protons, ester methyl protons, terminal methyl carbon atoms, and ester carbonyl carbon atoms from ^1H and ^{13}C NMR spectra can be used to distinguish MOO1, MOO2, and MOO3. ^1H and ^{13}C NMR of the ozonide ring protons and carbons can distinguish the *trans* from the *cis* isomer. Figure 2 shows the 400 MHz ^1H and ^{13}C NMR spectra for the ozonide ring structures of a *cis* and *trans* mixture



SCHEME 2A. Suggested heterolytic fragmentation pattern for MOO3 through an acid-catalyzed decomposition pathway.



SCHEME 2B. Suggested homolytic fragmentation pattern for MOO3.



SCHEME 2C. Suggested fragmentation pattern for MOO1.

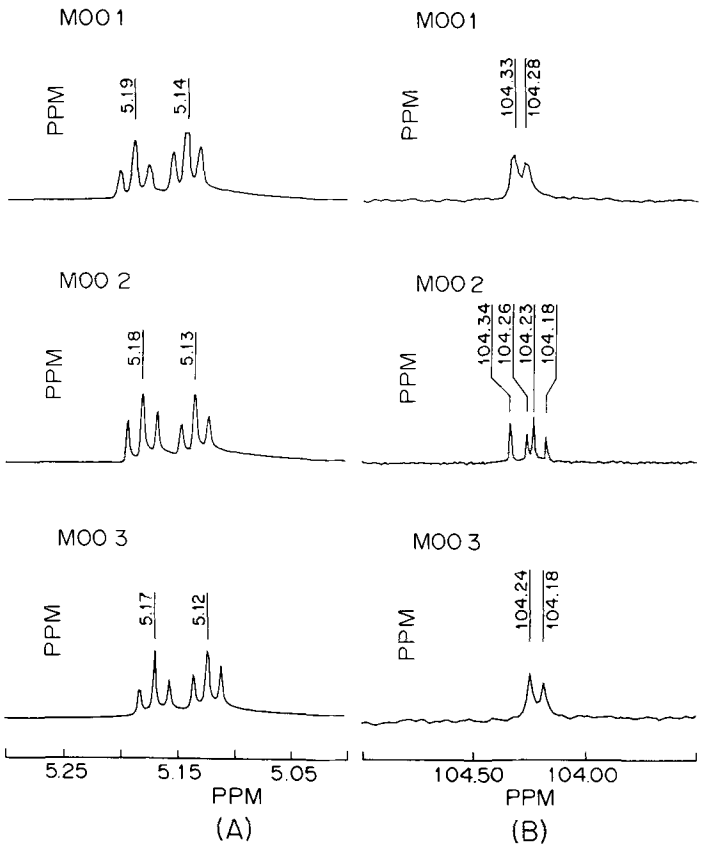


FIG. 2. The ^{1}H and ^{13}C NMR spectra for the ring protons of MOO1, MOO2, and MOO3 from the 400 MHz NMR spectrometer. In both ^{1}H and ^{13}C NMR spectra, the top one is for MOO1, the middle for MOO2, and the bottom for MOO3. A, ^{1}H NMR spectra; and B, ^{13}C NMR spectra.

of MOO1, MOO2, and MOO3. Both ^{1}H and ^{13}C NMR spectra of each individual isomer of either *cis*- or *trans*-MOO2 are reported elsewhere (21).

Assignments of the ozonide ring structures. From Table 3, the *trans* normal ozonide of methyl oleate (*trans*-MOO2) gives one triplet centered at δ 5.13 ppm (J = 4.8 Hz); the *cis* normal ozonide of methyl oleate (*cis*-MOO2) gives one triplet at δ 5.18 ppm (J = 5.2 Hz); and a mixture of the *trans* and *cis* isomers of MOO2 gives a set of two well resolved triplets at δ 5.13 ppm (J = 4.8 Hz) and δ 5.18 ppm (J = 5.2 Hz) (Fig. 2), which are not resolved on the 200 MHz NMR spectrometer (21). Previous studies only observed either δ 5.1 ppm for all six isomers (2) or δ 5.17 ppm (q) for MOO2) (20). Our assignments are consistent with the other physical properties of the ozonides based on the dipole moments (3,32,33). R_f values in normal-phase TLC, and R_t values in reversed-phase HPLC (Table 1). Based on the data from *trans*- and *cis*-MOO2, we assign the peaks at δ 5.19 and δ 5.17 ppm to the *cis* isomers for MOO1 and MOO3, and the peaks at δ 5.14 and δ 5.12 ppm to the *trans* isomers for MOO1 and MOO3, respectively (see Fig. 2A).

Since both MOO1 and MOO3 show either C-2 (for their *trans* isomers) or sigma (for their *cis* isomers) symmetry, the two ring carbons C-3 and C-5 (see Scheme 1) are equivalent in these cases. We observed two peaks at about δ 104 ppm for both MOO1 and MOO3 (Fig. 2B) due to the ring carbons; these peaks arise from the *trans* and *cis* isomers, not from carbons C-3 and C-5.

2D ^{1}H-^{13}C correlation experiments (21) supported these assignments. The results show that peaks at δ 104.34 and δ 104.23 ppm are produced by the *trans* isomers of MOO1 and MOO3 and that peaks at δ 104.26 and δ 104.18 ppm are produced by the *cis* isomers of MOO1 and MOO3, using the known values of the ring proton peaks of the corresponding *trans* and *cis* isomers. Thus, symmetry considerations, ^{13}C NMR, and 2D ^{1}H-^{13}C correlation experiments give consistent assignments for the ring carbons of MOO1 and MOO3.

We assigned the ^{13}C NMR peaks for carbons C-3 and C-5 in *trans*- and *cis*-MOO2 as follows. The structural environment of C-3 in *trans*- and *cis*-MOO2 is closer to that of C-3 (or C-5) in MOO3 rather than in MOO1, and the environment of C-5 in *trans*- and *cis*-MOO2 is closer to that of C-5 (or C-3) in MOO1 rather than in MOO3. Thus, we assigned δ 104.23 (*trans*-MOO2) and δ 104.18 ppm (*cis*-MOO2) for C-3 and δ 104.34 (*trans*-MOO2) and δ 104.26 ppm (*cis*-MOO2) for C-5 in MOO2. Most ^{13}C NMR signals for MOO2 agree with the results obtained by Ewing *et al.* (20,21). However, while Ewing *et al.* (20) detected only one peak at δ 106 ppm for the MOO2 ring carbons, we detected four MOO2 ring carbon peaks between δ 104.18 and δ 104.34 ppm. Complete assignments for the ozonide ring structures, including the terminal methyl, ester methyl, and ester carbonyl groups, are listed in Table 3. The NMR data for methyl oleate are also included in Table 3 for comparison.

Comparison of 400 MHz and 200 MHz NMR spectra. The 200 MHz ^{1}H NMR spectra of MOO1, MOO2, and MOO3 show an unresolved multiplet peak (partial-overlapped five lines) for the ring protons of both *cis* and *trans* isomers (21). The ^{13}C NMR spectra obtained at 50 MHz showed only one line for the ozonide ring carbons of both MOO1 and MOO3, and two lines for MOO2 (21). Thus, the AC-200 NMR spectrometer is not powerful enough to resolve these peaks, which may explain why previous work (2,20) gave incomplete assignments.

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Lipids of the Earthworm *Lumbricus terrestris*

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The lipid composition of the earthworm *Lumbricus terrestris* has been reexamined under conditions intended to avoid enzymatic and chemical alterations during storage, extraction, and fractionation procedures. The simple lipids included aliphatic hydrocarbons, steryl esters, glycerides, and at least nine different sterols, all thought to be derived from the diet. Free fatty acids, previously considered to be major components of worm lipids, comprised only 0.3% of the total lipid weight. Phospholipids included (in order of relative abundance) phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, as well as sphingomyelin. Glycolipids included cerebrosides and sulfatides containing both glucose and galactose, and gangliosides containing glucosamine and sialic acid. The fatty acid compositions of these lipid classes appeared to be a mixture of what are considered typical plant, bacterial, and animal acids. Several fatty acids found in the worms, including *cis*-vaccenic and eicosapentaenoic acids, were essentially absent from the dietary components, and it is concluded that these acids were synthesized in the worms. The earthworm derives much of its lipid adventitiously, but exerts at least some control over its tissue lipid composition.
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Earthworms are the major biomass in soil, constituting from 356 to 678 pounds per acre of arable land (1), and are widely used as a source of food and as an antipyretic (2,3). Charles Darwin estimated that the earthworms in an acre of land pass from 10 to 18 tons of earth and leaves through their intestines every year. In spite of their importance to agriculture, we know relatively little about the chemical composition of earthworms.

The general lipid composition of earthworms was reported by Lovern in 1940 (4), but the species was not identified. The neutral lipids of earthworms, especially the sterols, have been the subjects of considerable study (5–8), but the methodologies available at the time lacked sufficient resolving power for complete characterization. The few studies that have been made of earthworm phospholipids and glycolipids (8,9), while indicating a phospholipid class composition typical of animal species, have concluded that the glycolipids are very minor components and of limited variety.

The fatty acid composition of earthworm lipids is known to be quite complex (5,10,11). However, since high-resolution capillary column chromatography was not used in previous studies, it remained possible that the fatty acid distribution was even more complex than that reported.

These early studies of earthworm lipids were performed using worms dug from the soil at the time of analysis, with no attempt being made to control or characterize the dietary material or other environmental factors. The present work was undertaken in order to provide information on the lipid composition of the earthworm *Lumbricus terrestris* when the animals were maintained in the laboratory under controlled conditions, and precautions were taken to minimize the possibility for alterations before and during extraction and fractionation.

MATERIALS AND METHODS

Young earthworms (*L. terrestris,* "night crawlers"), sphagnum moss-based worm bedding, and worm feed (primarily based on corn meal) were obtained from Carolina Biological Supply Co. (Raleigh, NC). The bedding was extracted twice with chloroform and dried in a fume hood to ensure freedom from nematodes, to remove lipid-soluble polymeric material, and to delay the growth of mold. Worms were maintained at 12°C in glass chambers partially filled with moist bedding, and were fed a mixture of one part commercial worm feed, one part dried rabbit feces and two parts fresh coffee grounds (12). The chambers were in a dim light/dark cycle of 10 hr/14 hr. The worms were maintained in our laboratory for between three and six months after purchase.

The intestinal contents of the worms were partially voided by placing the worms for 24 hr in a chamber containing water and clean quartz gravel so they could be in or out of the water as they pleased. Worms taken for analysis weighed between 4 and 8 g, and had been obtained in the Spring of 1990. The worms were dropped into liquid nitrogen, and extracted while frozen.

Extraction involved the use of a stainless steel Waring blender canister to rapidly homogenize the worms in 20 volumes (v/w) of chloroform/methanol (2:1, v/v) at room temperature. This extract was filtered with suction through glass fiber filter paper (Reeve-Angel, Clifton, NJ) and the filter pad was reextracted with half the amount of chloroform/methanol used originally. The second extract was filtered through glass fiber paper and combined with the first. The combined extract was washed with 0.2 volumes of 1.2% aq. KCl by the method of Folch *et al.* (13). The lower phase was rotary evaporated under vacuum at 20°C, and the residue immediately weighed and fractionated. Results reported in this paper are the means for three batches of worms, five worms per batch, unless stated otherwise.

Fractionation of lipid classes. All solvents used in this study were (HPLC) grade, either MCB OmniSolv (EM Science, Cherry Hill, NJ) or glass distilled (Burdick & Jackson, Muskegon, MI). The extracted lipids were first separated into major classes by chromatography on columns of Unisil silicic acid (Clarkson Chemical Co., Williamsport, PA) prepared as hexane slurries. The

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Abbreviations: CL, cardiolipin; ECL, equivalent chain length; GLC, gas-liquid chromatography; HPLC, high-pressure liquid chromatography; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; TLC, thin-layer chromatography; TMS, trimethylsilyl.

loading factor was 15 mg of lipid per g of adsorbent. Simple lipids were eluted as Fraction I with chloroform (20 mL/g silicic acid). Glycolipids were then eluted as Fraction II with chloroform/acetone (1:1, v/v), 10 mL/g silicic acid, and Fraction III with acetone, 10 mL/g. After a wash with chloroform (10 mL/g silicic acid) that did not elute additional lipid, phospholipids were eluted with methanol (10 mL/g silicic acid). Recovery averaged 95.6% of the weight of extract loaded onto the columns. All lipid fractions were stored under nitrogen without solvent at −20°C until processed further.

The simple lipid fraction (I) was examined by thin-layer chromatography (TLC) on 250 micron silica gel GF plates (Analtec, Inc., Newark, DE) using (a) hexane/benzene (9:1, v/v); and (b) hexane/diethyl ether/acetic acid (85:15:1.5, v/v/v) as developing solvents. Detection was with iodine vapors and by charring with sulfuric acid/nitric acid/water (5:4:3, v/v/v). Aliphatic hydrocarbons and steryl esters were separated from portions of Fraction I by chromatography on Florisil as described by Carroll (14).

Glycolipids (Fractions II and III) were examined by TLC in (c) chloroform/methanol/water (60:35:8, v/v/v) (15). The α-naphthol spray of Siakotos and Rouser (16) was used to detect carbohydrate-containing components. The hypochlorite-benzidine spray of Bischel and Austin (17) was used to detect sphingosine, and the resorcinol spray described by Svennerholm (18) was used to detect sialic acid. A molybdate spray was used to visualize phospholipid contaminants (19).

Phospholipids were separated by two-dimensional TLC on plates that had been pre-developed in acetone before activation. Plates were first developed in (d) chloroform/methanol/acetic acid/water (50:25:8:4, by vol.), dried, and developed in the second dimension in (e) diisobutyl ketone/acetic acid/water (8:5:1, v/v/v) using lined tanks. Fractions were detected with iodine vapors, scraped off, and analyzed for lipid phosphorus by the method of Bartlett (20). Corresponding regions were scraped from solvent-developed, but otherwise blank, plates to serve as background controls. Phospholipid class identifications were confirmed by one-dimensional TLC using chloroform/methanol/14% aq. ammonia (68:28:8, v/v/v). These plates were sprayed with molybdate for lipid phosphate, ammonia/silver nitrate for reducing lipids, ninhydrin for amino groups, and Dragendorff's reagent for choline-containing lipids (21). Identification was based on cochromatography with standards and appropriate color reactions with the selective spray reagents.

Reference standards for TLC included phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and mixed gangliosides (bovine origin), phosphatidylinositol (PI) and phosphatidylglycerol (PG) (soybean) were from Supelco (Bellefonte, PA); lysoPE (LPE), LPC, LPS, sphingomyelin (SPH), brain sulfatides and cerebrosides (bovine) were from Applied Science Laboratories (State College, PA), and cardiolipin (CL) from ICN Pharmaceuticals (Cleveland, OH).

Portions of Fraction V were chromatographed on columns of Silica Gel 60 (Brinkmann, EM Laboratories, Elmsford, NY) chloroform slurry. After a wash with 4 mL of chloroform per g of silicic acid, amino-phospholipids (mostly PE) were eluted with 7.5 mL of chloroform/methanol (4:1, v/v) per g and zwitterionic phospholipids (mostly PC) were eluted with 7.5 mL of chloroform/methanol (1:4, v/v) per g.

Other analytical methods. Glycolipids were quantified by the colorimetric anthrone assay following hydrolysis in ethanol/chloroform/2N HCl (22). Sterols and steryl esters were measured by a ferric chloride procedure that gives the same molar response for free and esterified sterols (23). Aliphatic hydrocarbons were estimated by weighing the residue from the hexane fraction off Florisil. Intrared spectra of thin films on salt plates or pressed KBr pellets were obtained using a Perkin-Elmer model 1320 Infrared Spectrophotometer (Perkin-Elmer, Norwalk, CT). UV/visible spectra were scanned on a Beckman DU-7 instrument (Beckman Instruments Co., Fullerton, CA). Plasmalogens were determined by the method of Gottfried and Rapport (24). Free fatty acids were quantified using the rhodamine assay of Chakrabarty *et al.* (25).

Glycolipids were hydrolyzed for analysis of the carbohydrate moieties after reflux for 2–3 hr in *tert*-butanol/water (2:1, v/v) containing 2N HCl. The fatty acids and alcohol were removed by adding 6 volumes of water and extracting three time with diethyl ether. The aqueous phase was frozen in a dry-ice/acetone bath and lyophilized prior to derivatization for gas chromatography. This is a modification of the established method of Radin *et al.* (22), in which substitution of *tert*-butanol for ethanol avoids the formation of alkylated artifacts while retaining the ability to dissolve lipids. The modified procedure gave complete release of sugars from standards of bovine brain sulfatides (data not shown).

Total sterols were obtained by saponifying the simple lipid fraction in 50% aqueous ethanol containing 2N KOH under reflux for 90 min. After adding water to reduce the ethanol to 25%, non-saponifiables were extracted twice with 5% diethyl ether in hexane. The aqueous phase was acidified with HCl and extracted with diethyl ether to recover the fatty acids.

Squalene, cholesta-3,5-diene, cholesteryl oleate, and the reference sterols cholesterol, stigmasterol, lanosterol, ergosterol, epicholesterol, β-cholestanol, desmosterol, β-sitosterol, campesterol, dihydrobrassicasterol, 7-dehydrocholesterol, and cholest-7-en-3β-ol were from Sigma Chemical Co. (St. Louis, MO). Reference sterols coprostanol, fucosterol, and brassicasterol were from Supelco.

Lipid fractions were converted to fatty acid methyl esters using BF_3/methanol (26) directly, except for the simple lipids, in which only the saponifiables were derivatized in order to prevent the formation of BF_3/sterol artifacts. Free sterols were converted to trimethylsilyl (TMS) derivatives in methylene chloride/methyl trimethylsilyl trifluoroacetamide (3:1, v/v) by heating for 15 min at 60°C. TMS derivatives of the monosaccharides derived from hydrolysis of glycolipids were made using Tri-Sil Z (27). Hydroxy-acid methyl esters were converted to TMS derivatives with *bis*-(trimethylsilyl) trifluoroacetamide/methylene chloride (1:2, v/v) at room temperature. All silylation reagents were obtained from Alltech Associates (Deerfield, IL).

Gas-liquid chromatography. All separations were performed using a Varian model 3700 gas chromatograph with hydrogen flame ionization detection and splitless injection (Varian Associates, Inc., Palo Alto, CA). Quantification (molar percentages) was based on peak areas as reported by a Hewlett-Packard model 3390A electronic integrator (Hewlett-Packard, Cupertino, CA).

TMS derivatives of sterols were chromatographed on a 25 m × 0.25 mm fused silica capillary column

containing a 0.2-micron layer of RSL-150 methyl silicone (Alltech, Deerfield, IL) at 250°C, with helium as carrier gas at 20 psig. Identifications were confirmed on a more polar column, 25 m × 0.25 mm, coated with a 0.2-micron layer of DB-1701 (Alltech), at 200°C. In addition, the TMS derivatives were examined by GLC/mass spectrometry on a Kratos Concept 1SQ mass spectrometer (Kratos Analytical, Ramsey, NJ) using the 70-eV electron impact ionization mode. Samples were introduced through a 30 m × 0.25 mm DB-5 capillary column programmed from 150–250°C at 4°C/min.

Fatty acid methyl esters were determined on a 25 m × 0.25 mm fused silica capillary column coated with RSL-950 (Alltech, equivalent to SP 2340), cyanosilicone, at 140°C. Identifications were based on direct cochromatography with standards, confirmed by cochromatography on the methyl silicone column described above and, where feasible, reconfirmed on a packed column of 5% Apiezon L on 100/120 mesh Gas Chrom Q, 2 m × 3 mm, with helium as carrier at 30 mL/min at 205°C.

TMS derivatives of hydroxyacid methyl esters were analyzed on the methyl silicone column with a temperature program from 150 to 250°C at 2°C/min. Identifications were confirmed by GLC/mass spectrometry using a 15 m × 0.25 mm DB-5 column programmed from 40–325°C at 10°C/min. Electron impact ionization (70 eV) mass spectra were generated as for the sterols above.

Reference fatty acid methyl esters included all those (normal, branched, hydroxy, cyclic) available from Applied Science Laboratories, Supelco, Sigma, and Analabs, Inc. (North Haven, CT). Additional confirmation of the identifications of the fatty acid methyl esters involved chromatography before and after catalytic hydrogenation over platinum oxide in n-heptane, and separate analysis of the reconstituted methyl esters after separation of the mercurimethoxy derivatives according to the number of double bonds (28).

RESULTS

Six worms were extracted individually, giving a mean lipid content of 1.23% ± 0.20% (S.D.) of worm fresh weight. The extractable lipid from three batches of worms was distributed among chromatographic fractions as shown in Table 1.

Simple lipids. Aliphatic hydrocarbons were quantified by weight only, since the composition of this fraction has been examined in great detail by GLC/mass spectrometry previously (29). Squalene was not detected on TLC in solvent (a); if present, it must have amounted to less than 1% of the lipid. The data in Table 1 for sterols and steryl esters are lower limits, as some of the minor components do not give a strong color reaction with ferric chloride. Free fatty acids were extremely low, probably because of the precautions taken to prevent artifactual hydrolysis. If the lipid extract was stored in chloroform for one month at −20°C prior to fractionation, most of the PE and at least part of the PC were converted to lyso forms with a major increase in free fatty acids (data not shown).

Glycerides were the major components in the simple lipid fraction. TLC showed that most of the glyceride was a triglyceride, with a barely detectable level of diglyceride also being observed.

TABLE 1

Lipid Class Composition of *L. terrestris*[a]

Lipid fraction	Total lipid (%) ± S.D.
Simple Lipid (Fr. I)	(29.0 ± 1.5)
Hydrocarbons	1.25 ± 0.05
Steryl esters	3.0 ± 0.10
Free fatty acids	0.32 ± 0.01
Sterols	3.75 ± 0.08
Glycerides	20.7 ± 1.1
Glycolipid (Fr. II)	(6.6 ± 0.3)
Glycolipid (Fr. III)	(4.8 ± 0.4)
Phospholipid	(59.5 ± 1.3)
Phosphatidylethanolamine	19.8 ± 0.6
Phosphatidylcholine	23.8 ± 0.7
Phosphatidylserine	4.6 ± 0.14
Phosphatidylinositol	4.0 ± 0.12
Sphingomyelin	2.1 ± 0.06
Lysophosphatidylethanolamine	3.3 ± 0.1
Lysophosphatidylcholine	2.1 ± 0.07

[a]Results are expressed as weight percentage of total extractable lipids. SD, standard deviation between batches. Numbers in parentheses are for the weights of the fractions from Unisil silicic acid chromatography. Phospholipid values based on phosphorus × 25. Sterols and free fatty acids based on colorimetric assay. Glycerides by weight difference. Extractable lipid amounted to 1.23 ± 0.20% of the worm fresh weight (average of six determinations).

Sterols. Table 2 shows the distribution of sterols found after saponification and trimethylsilylation. It was not possible to distinguish between R- and S-isomers of the 25-alkyl-substituted sterols. For example, the component labeled brassicasterol could also include its isomer crinosterol. Except for these optical isomers, which are not resolved under GLC conditions, resolution of the TMS derivatives was excellent and much better than we observed for the free sterols (not shown). Ergosterol was lost if the lipid extract was stored without protection from light, although it was much more stable after saponification and isolation of the nonsaponifiables.

Glycolipids. The two glycolipid fractions from the Unisil columns could be fractionated cleanly by TLC if the extract was processed while fresh. If build-up of free fatty acids through spontaneous hydrolysis on storage was allowed to occur, the glycolipid fractions smeared badly. Most of the dark pigment in the lipid extract eluted in Fraction II with chloroform/acetone (1:1, v/v). This material is not lipid, even though it is lipid soluble, and it has not been characterized in the present study.

Fraction II contained material chromatographing (TLC) with bovine cerebrosides. This material gave a double spot, as is always seen when the fatty acids include a mixture of normal and hydroxylated forms (30). These spots were positive for carbohydrate with the naphthol spray, and positive for sphingosine with the hypochlorite-benzidine reagent. Hydrolysis and GLC analysis of the TMS derivatives gave only glucose and galactose in a ratio of 2.39 to 1, indicative of a mixture. In contrast, similar treatment of bovine cerebroside and sulfatide gave only galactose. Fraction II also contained a very slight amount of carbohydrate/sphingosine positive material chromatographing with sulfatide, as well as a small amount of sterol carryover from Fraction I.

TABLE 2

Distribution of Total Sterols[a]

Sterol	RRT[b] RSL150	Area %, TMS deriv.	Characteristic mass peaks (m/z) [% rel. abundance]
Cholesterol	1.000	66.9 ± 2.8	458 [100], 443 [15], 368[87], 353 [32], 329 [94], 129 [71]
β-Cholestanol	1.036	1.7 ± 0.3	460 [100], 445 [17], 370 [85], 331 [16], 129 [39]
Desmosterol	1.092	8.6 ± 1.4	456 [92], 441 [41], 366 [45], 343 [87], 327 [57], 129 [100], 60 [99]
Brassicasterol	1.118	8.8 ± 0.6	470 [82], 455 [8], 380 [36], 341 [19], 329 [8], 255 [27], 129 [41], 71 [100]
Ergosterol	1.200	3.1 ± 0.3	468 [100], 453 [7], 378 [15], 363 [83], 337 [45], 253 [20], 69 [52]
Campesterol	1.288	6.3 ± 0.9	472 [100], 457 [15], 382 [74], 367 [25], 343 [80], 129 [61]
Stigmasterol	1.388	3.6 ± 0.2	484 [56], 469 [6], 394 [24], 379 [6], 343 [15], 255 [23], 100 [100]
β-Sitosterol	1.582	1.0 ± 0.1	486 [100], 471 [11], 396 [71], 381 [22], 357 [74], 129 [64], 70 [37]
Isofucosterol	1.634	0.2 ± 0.2	Tentative, much background interference.

[a]Nonsaponifiable lipids derivatized with methyl trimethylsilyl trifluoroacetamide in methylene chloride and analyzed by gas chromatography on a 25-m capillary column coated with methyl silicone (RSL-150), under isothermal conditions. Identifications confirmed on a 25-m capillary column of DB 1701 and by GC/mass spectrometry (EI-70 eV). For details, see Materials and Methods.

[b]RRT, retention time relative to that of cholesterol TMS, on the RSL-150 capillary column.

Fraction III contained significant amounts of both material chromatographing with sulfatide and material chromatographing with bovine gangliosides (double spots in both cases). The latter, but not the former, reacted positively with the resorcinol-copper sulfate reagent for sialic acid, as well as the naphthol and hypochlorite-benzidine reagents. Refluxing for 2 hr in 66% *tert*-butanol containing 2N HCl hydrolyzed glycosidic bonds, but did not hydrolyze the amide bonds of standard *N*-acetyl-glycosamine or *N*-acetyl-neuraminic acid. Fraction III gave glucose, galactose, *N*-acetyl-glucosamine, and sialic acid in an apparent ratio of 2.29:1:1.69:1.02. This mixture confirmed the presence of gangliosides in Fraction III.

Infrared spectra of Fractions II and III showed amide at ~1645 cm^{-1}, S=O at 828, and S-O at ~1368 and/or 1182 cm^{-1}, as well as strong -OH absorption in the 3300-3400 cm^{-1} region. This confirmed the presence of sulfatides in both fractions (31).

The anthrone assay for hexoses indicated that Fraction II contained approximately 390 nmole of hexose per mg of fraction weight, with 720 nmole hexose per mg in Fraction III. Amino sugars do not contribute to this assay, so Fraction III would have contained a total of 1.3 micromoles of monosaccharide-equivalent per mg of fraction weight. If we assume a mean molecular weight of 760 for typical cerebrosides, and assume that sulfatides are minor components in Fraction II, then it is possible to estimate that cerebrosides amounted to approximately 1.9% of the total lipid weight (upper limit). Both glucose- and galactose-containing lipids would be included in this estimate. The bulk of the weight of Fraction II not included in glycolipid was due to pigment (almost absent from Fraction III).

We were not able to estimate a mean molecular weight for Fraction III glycolipids. Moreover, the exact proportion of sulfatide to ganglioside is not known for these preparations. Therefore, we can only report that sulfatides plus gangliosides comprised approximately 4.6% of the total extractable lipid. We cannot state that glycosyl glycerides were absent, but all the naphthol-positive spots we detected were also positive for sphingosine.

Phospholipids. The phospholipid distribution is also shown in Table 1. The two major classes were PC and PE, with PS, PI, SPH, and lyso forms as minor components. CL, PG, and phosphatidyl-*N*-methylethanolamine were not detected, although they could have been present below our limits of detection. Phosphate assays indicated that Unisil fraction V could not have contained significant amounts of material lacking lipid phosphorus (not shown). Thus phospholipid was the most abundant class of extractable lipids in these worms (about 60% of the total lipid by weight).

The iodination assay (23) indicated that only 3.7% of the phospholipid was present in the form of plasmalogen (vinyl ether). We did not assay for saturated ether-containing lipids.

Fatty acids. The fatty acid compositions of various lipid fractions are summarized in Tables 3 and 4. Infrared spectra of the methyl ester preparations showed no indication of *trans* double bonds, keto groups, or epoxides. Traces of dimethylacetals were indicated by small peaks at 1195 and 1122 cm^{-1} (32). Very low levels of the dimethyl acetals of palmitaldehyde and stearaldehyde were detected in the methyl ester preparations derived from the total phospholipids (not shown). Hydroxyacids were clearly present in the phospho- and glycolipid fractions, and were separated from the simple fatty acids (as methyl esters) on silicic acid (33). The individual hydroxyesters were identified on the basis of their GLC retention parameters before and after reaction with *bis*(trimethylsilyl) trifluoroacetamide (34,35), and confirmed by GLC/mass spectrometry. In no case were hydroxy acids other than 2-hydroxy acids detected. We particularly looked for ricinoleic acid, but did not find it. All of the hydroxyacid methyl esters (as TMS derivatives) gave mass spectra in which the base peak was either *m/z* 73 or the ion corresponding to M-59 (loss of $COOCH_3$), with only a very weak (~1%) molecular ion, a strong peak at M-15, and a minor peak at M-31. The spectra closely matched those published for the 2-hydroxyacid methyl ester TMS derivatives from brain (35).

The complexity of the fatty acid mixtures is apparent from Table 3. Acids typical of animals (arachidonic, other C_{20} polyenes) were abundant, as were characteristic plant acids (pristanic and phytanic), and bacterial acids (vaccenic, branched saturated). It was not possible to

TABLE 3

Fatty Acids of Worm Lipids[a]

Acid[b]	ECL	Percentage of non-hydroxylated fatty acids						
		Steryl esters[c]	Glycerides	Total phospholipid	Amino lipids	Crude PC	Glycolipid II	Glycolipid III
<14	<14	1.14	0.97	—	0.55	0.36	—	—
n-14:0	14.00	—	2.13	0.97	0.56	0.63	1.10	—
a-15:0[d]	14.44	5.25	9.38	2.22	0.76	0.28	5.13	—
n-15:0	14.97	1.71	0.68	0.84	2.35	2.55	—	
n-15:1	15.39	—	0.89	—	2.76	—	5.55	—
i-16:0	15.48	—	1.95	1.25	—	9.43	—	2.40
Pristanate	15.69	0.85	0.94	0.21	—	—	—	—
n-16:0	16.00	26.05	12.29	2.96	9.88	8.97	20.29	11.74
9-Me-16	16.13	—	—	—	—	4.40	—	—
n-16:1	16.39	—	—	0.10	1.20	2.20	0.44	—
a-17:0	16.48	0.56	0.522	1.93	1.12	0.49	1.90	3.31
br-17:0	16.63	0.39	2.18	1.00	0.52	0.75	2.12	2.19
Phytanate	16.97	1.37	2.97	3.05	1.82	3.47	4.03	7.25
n-16:2	17.11	—	—	0.12	0.56	—	—	—
9-cyc-17	17.24	6.04	1.35	0.11	—	0.30	—	—
i-18:0	17.45	0.88	2.57	0.82	2.30	6.02	9.42	3.23
Unknown	17.86	4.68	—	—	1.14	3.21	1.37	—
n-18:0	18.00	39.55	11.08	7.17	9.37	9.69	12.24	34.88
11-Me-18	18.23	—	—	—	0.99	—	—	—
C-18:1Δ9	18.41	2.61	11.25	4.46	3.56	0.38	8.45	3.47
C-18:1Δ11	18.49	3.07	6.76	9.75	2.28	1.92	5.35	6.84
Unknown	18.93	—	—	0.73	—	0.52	—	—
n-18:2Δ9	19.15	0.47	12.09	7.85	16.46	0.99	7.54	5.80
11-cyc-19	19.32	—	—	0.29	—	0.36	—	—
18:3/20:0	20.00	—	1.35	2.02	7.34	1.07	—	—
n-20:1Δ11	20.32	0.93	2.19	9.61	7.83	11.79	6.09	7.80
n-20:2	20.79	—	8.71	1.26	—	0.97	—	—
20:2/21:0	21.00	—	0.77	3.22	13.37	0.97	4.73	4.32
13-cyc-21	21.24	—	—	1.47	—	T	1.72	—
n-20:3	21.60	—	T	1.81	—	0.90	—	—
n-30:4Δ5	21.86	T	0.90	15.22	9.83	17.24	—	6.80
n-22:1	22.35	—	—	0.96	T	T	—	—
n-20:5Δ5	22.74	—	0.36	14.48	5.01	9.99	—	—
n-22:2	23.11	—	—	1.02	—	—	—	—
n-22:4	23.80	T	—	1.21	—	T	—	—
n-24:0	24.00	—	T	0.51	—	T	T	—
n-22:5	24.65	—	T	1.52	—	T	—	—

[a] Fatty acid methyl esters analyzed by capillary GLC on RSL-950 at 140°C. All identifications are based on cochromatography with known standards. The identifications were confimred on RSL-150 and Apiezon L columns. ECL, equivalent chain length on RSL-950 column. Analytical data are presented as percentage of non-hydroxylated fatty acids in a given fraction for those acids comprising at least 0.1% of the total. A "T" indicates that at least one of the three batches analyzed had 0.1% of that component.

[b] Fatty acids designated as number of carbon atoms:number of double bonds. n, Normal; i, iso; a, anteiso; br, unidentified branching pattern; cyc, cyclopropane ring in chain at the specified location; Me, branch methyl group at specified location. "Unknown" indicates a component for which we lacked a matching standard.

[c] Fractions from Unisil silicic acid columns or (steryl esters) from Florisil. See Materials and Methods for details.

[d] This peak includes trimethyltridecanoate with the anteisopentadecanoate.

rationalize the fatty acid compositions solely on the basis of the worm diet (Table 5). None of the dietary components contained more than a trace of vaccenic acid, yet vaccenic acid equaled or exceeded oleic acid in the worms. The worms contained branched acids and hydroxy acids that were not detected in the diet. One of the most abundant fatty acids in the worms, eicosa-11-enoic acid, was no more than a trace component of the diet.

Steryl esters contained primarily saturated fatty acids, with less than 1% being polyunsaturated. The glycerides had a fatty acid composition similar to that of the mixed diet, except for increased amounts of branched saturated acids. Linoleic acid was high in the glycerides, but C_{20} polyenes were almost absent. The phospholipids contained C_{20} polyenes as the main components, with fatty acid compositions more reminiscent of animal than of plant lipids. At the same time, the high levels of vaccenic acid, branched acids, and cyclopropanoid acids in the phospholipids suggested a partial bacterial origin.

DISCUSSION

The lipid content of *L. terrestris* reported here, 1.23 ± 0.20% of the worm fresh weight, may be compared to the 1.2–2.5% reported by Hansen and Czochanska (10), 0.65–3.5% (average 1.25%) reported by Cerbulis and Wight

TABLE 4

Hydroxylated Fatty Acids of Worm Lipids[a]

Acid[b]	ECL[c]	Percentage of total 2-hydroxyacids[d]		
		Total phospholipids	Glycolipids Fraction II	Glycolipids Fraction III
16:0	17.86	6.20	2.95	19.17
17:0	18.95	11.46	2.37	16.52
18:0	19.99	5.26	0.42	—
19:0	20.87	4.31	—	—
20:1	21.51	—	0.21	1.10
20:0	21.83	5.47	10.38	—
21:1	22.50	9.04	2.25	14.71
22:1	23.44	—	0.83	9.48
22:0	23.81	5.89	1.89	10.41
23:1	24.45	—	11.84	—
23:0	24.95	7.99	0.40	—
24:1	25.45	7.88	1.85	—
24:0	25.93	7.89	2.48	1.15
25:0	26.95	—	11.86	—
26:1	27.35	—	15.69	27.49
26:0	27.82	18.09	34.76	T
27:0	28.93	3.15	T	T
28:1	29.30	5.26	T	T
28:0	29.87	2.09	T	T

[a]Separated from simple fatty acid methyl esters on Unisil. Analyzed as TMS derivatives on RSL-150, identifications were confirmed on DB 1701, and by GLC/mass spectrometry.

[b]Number of carbon atoms:number of double bonds. Hydroxyl groups in 2-position.

[c]ECL, equivalent chain length.

[d]2-Hydroxy acids amounted to 4.5% of the total fatty acids from the phospholipids, 20.5% of the fatty acids from glycolipid Fr. II, and 7.6% of the fatty acids from Fr. III (weight basis). T, trace (less than 0.1%).

TABLE 5

Fatty Acid Composition of Worm Feed[a]

Fatty acid[b]	Percentage of total fatty acids		
	Commercial feed	Coffee grounds	Rabbit feces
<16:0	0.19	0.11	19.50
16:0	20.55	37.51	29.39
Pristanate	0.46	—	1.12
16:1 (9)	0.35	0.11	0.51
Phytanate	0.15	—	1.09
Branched 17	1.04	—	5.85
18:0	3.41	6.98	10.27
18:1 (9)	33.25	7.86	15.15
18:1 (11)	—	—	0.40
18:2 (9)	37.51	42.52	12.59
18:3 + 20:0	1.79	3.80	3.88
20:1 (11)	0.72	0.25	0.38
20:2	0.44	—	trace
20:3	trace	—	—
20:4 (5)	trace	—	trace
22:0	trace	—	—

[a]Total lipid content of feed mixture was 42 mg/g.

[b]Number of carbon atoms:number of double bonds (Δ position of first double bond, if known).

Taylor (5), and the 1.2–1.3% reported by Lovern (4). The lipid content reportedly depends on the season of the year in which the worms are collected (10,11) which, in turn, may depend upon the organic composition of the soil at those seasons. If that is the case, then the present data for worms maintained on a fixed diet may be independent of the season in which the worms were procured.

Cerbulis and Wight Taylor (5) and Nooner *et al.* (29) reported that aliphatic hydrocarbons amounted to 3% of the total lipid weight. Our finding hydrocarbons to comprise only 1.25% of the total lipid weight may reflect the fact that the worm bedding was free of organic extractables in our study, while the worms studied previously (5) were collected directly from the soil just prior to analysis.

Lovern (4) and Cerbulis and Wight Taylor (5) had reported that earthworm lipids did not contain triglycerides. The absence of glycerides was later shown by Hansen and Czochanska (36) to be an artifact resulting from cold storage of dead worms prior to extraction. It would not be surprising for hydrolysis of lipids to have also occurred during the extremely prolonged (four days) room temperature extraction procedure of Cerbulis and Wight Taylor (5). However, it was still accepted that earthworm lipids were rich in free fatty acids (10,11). We found that storage of the extracted lipids at −20°C, for even a few days prior to fractionation, resulted in decomposition of the phospholipids (especially PE) to give lysophosphatides and free fatty acids. Under the conditions of extraction and immediate fractionation described here, free fatty acids were not seen on TLC of the lipids, and were only detectable (at 0.3% of the total lipid) by colorimetric assay. However, it is also possible that the soil in which the worms were living contained a high level of free fatty acids in the earlier studies.

Previous reports that ergosterol was the second most abundant sterol in the earthworm, amounting to 23% or more of the sterol fraction (see references in 4), were based on methods incapable of separating the wide variety of sterols actually present. Cerbulis and Wight Taylor (5) observed the presence of at least six different sterols in *L. terrestris*; however, gas chromatography of the free sterols on a packed column did not provide sufficient resolution to permit quantification. We can confirm the presence of five of the six sterols reported previously (5), but we did not detect γ-sitosterol. We found ergosterol to be only the sixth most abundant sterol present, behind cholesterol, brassicasterol, desmosterol, campesterol, and stigmasterol. Four of the sterols found in the present study (dihydrocholesterol, desmosterol, brassicasterol, and isofucosterol) have not been reported previously in *Lumbricus*. Dihydrocholesterol and desmosterol are common animal sterols, and the former was reported to occur in *E. foetida* (8), as well as in the cricket *A. domesticus* (37). The compound here identified as desmosterol may correspond to what was thought to be 7-dehydrocholesterol on the basis of cochromatography (8). These two sterols are not separated on non-polar columns. However, the intense peaks at *m/z* 343 and 69 in the mass spectrum of this compound clearly show the second double bond to be at C-24 rather than C-7 (38). Brassicasterol occurs in *Mollusca* (39) and *Perichaeta* (40), and was tentatively identified in *Eisenia* (8). Campesterol, stigmasterol and isofucosterol are generally considered characteristic of plants. The relatively low level of ergosterol found in the present study

may reflect the low level of fungal metabolites available to our worms.

Since it has been shown by Wootton and Wright (41,42) that *L. terrestris* has a block in sterol biosynthesis such that mevalonic acid can be incorporated into squalene but not into sterols, and since common soil bacteria do not produce sterols (43), it must be assumed that the sterols found in earthworms are derived as such from the organic matter in their diet. It is not surprising, therefore, that many of the sterols found in the worms are typical plant sterols. No lanosterol or lathosterol was detected, again suggesting the absence of *de novo* synthesis of sterols in *Lumbricus.*

Phospholipids were reported to comprise 33–44% (4), 25–41% (5) or 59.5% (this study) of the total extractable lipid of *L. terrestris.* Hansen and Czochanska (11) reported 57.6% phospholipid in *L. rubellus* and *Allolobophora caliginosa* lipids. The two lowest values were from the studies in which hydrolysis of the lipids almost certainly had occurred prior to analysis, suggesting that the higher value is more likely correct. That the two most abundant phospholipids were PC and PE (in that order) was also the case for *Eisenia foetida* (8) and the purified chloragosomes of *L. terrestris* (44). *E. foetida* reportedly also contained PS (8), but not PI, while both PS and PI (but not SPH) were found in isolated nerve tissue from *L. terrestris* (9).

Okamura *et al.* (9) reported finding only a trace of glucocerebroside and no other glycolipids in the nerve tissue of *L. terrestris.* Hansen and Czochanska (11) reported that glycolipids (uncharacterized) comprised 2.3% of the extractable lipid from *L. rubellus* and *A. caliginosa.* Glycolipids were only trace components of the lipids of *E. foetida* (8), but phospholipids were also extremely low in this species (11%). It has been reported that the large amount of polar pigment extracted from earthworms can interfere in the detection of gangliosides (8). This pigment mixture is thought to consist of coumarols and flavonols (44). Since the pigment was separated from the sialic acid-containing lipids under our chromatographic conditions, we may have escaped the interference problem. Glycolipids, as determined both directly on a weight basis and by colorimetric and gas chromatographic determination of the monosaccharides after hydrolysis, amounted to at least 6.7% of the extractable lipid in the present study. This is a lower limit, since it is probable that some of the gangliosides would have been lost in the aqueous phase of the Folch extraction (45).

Detailed studies of the fatty acid compositions of total lipids, lipid fractions, or individual phospholipids were reported for *L. terrestris* by Cerbulis and Wight Taylor (5), for a mixture of *L. rubellus* and *A. caliginosa* by Hansen and Czochanska (10,11), and for nerve tissue from *L. terrestris* by Okamura *et al.* (9). Hydroxy fatty acids were not reported in any of these studies.

Early studies (5) indicated that *L. terrestris* contained fatty acids from C_{11} to C_{27} in length, with significant amounts of monoenes, traces of dienes, and no fatty acids with more than two double bonds except a C_{16} acid with four double bonds. Polyunsaturated acids were also not detected in *L. terrestris* nerve tissue (9). In contrast, polyunsaturated acids, especially arachidonic acid and a C_{20} acid with five double bonds ($\omega 3$), were major components in the lipids from *L. rubellus* and *A. caliginosa* (11). We found polyunsaturated fatty acids to be major components of the phospholipids of *L. terrestris,* suggesting that the prolonged extraction time (5) or extensive manipulation involved in isolating nerve tissue (9) may have permitted autooxidation to have occurred, depleting these components in the earlier studies of this species. We found no indication of acids with conjugated double bonds, keto groups, or epoxy groups in the present study. Table 3 shows only about half the fatty acids actually observed in this study; many others occurred, but at levels below 0.1% of the total (below our limit of reliable quantification).

The occurrence of simple branched and isoprenoid fatty acids reported here is in agreement with what was reported for *L. rubellus* and *A. caliginosa* (11). In combination with the major presence of *cis*-vaccenic acid, the cyclopropanoid C_{17} acid and the polyunsaturated C_{20} and C_{22} acids, these fatty acids suggest a combination of plant, bacterial, and animal origins. Since several of the major fatty acids, including vaccenic acid and eicosapentaenoic acid, could not be traced to the worm diet, it is necessary to conclude that fatty acid synthesis does occur in worms (possibly with help from the intestinal flora) and that worms do exert some control over the fatty acid composition of their lipids. This is consistent with the observations of Wootton and Wright (41), who found incorporation of radioactivity from ^{14}C-labeled mevalonic acid into fatty acids in *L. terrestris.*

Hydroxy fatty acids were found in the phospholipid fraction and in both fractions of glycolipids in the present study. Those in the phospholipid fraction may have been associated with sphingomyelin, although that component was not separately isolated. The hydroxy acids in the glycolipid fractions support the conclusion that the glycolipids did indeed include sphingolipids. This observation is in contrast to the results found for isolated *L. terrestris* nerve tissue, where sphingolipids were essentially absent (9). Whether the absence of sphingolipids in the studies of nerve tissues indicates that they are localized elsewhere, or whether the fact that the worms were killed with hot water and the extraction performed at 70°C (9) may have, in some way, prevented the recovery of sphingolipids is not known.

PE and PS from worm nerves were reported to contain high concentrations of plasmalogens (9). We found only traces of plasmalogens in the total worm phospholipids, suggesting that plasmalogens may be negligible components of *L. terrestris* lipids in tissues other than nerves.

It is clear that misleading results will be obtained for the lipid composition of worms if precautions are not taken to prevent enzymatic, chemical (hydrolytic), photochemical and oxidative alteration during storage of dead worms, extraction, and/or storage of the lipid extracts. These alterations seem to include extensive hydrolysis of triglycerides and PE, loss of polyunsaturated fatty acids, and destruction of photosensitive sterols. The remarkable complexity of the lipid mixture from earthworms can only be observed if such alterations are strenuously avoided.

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COMMUNICATION

Changes in Fatty Acid Composition of Phospholipids from Liver Microsomes and Nuclei in Rats Fed a Choline-Free Diet

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Male F-344 rats were fed a choline-free (CF) diet, and changes in phospholipid content, phospholipid fatty acids and phospholipase A_2 activity in liver nuclei and microsomes were examined during the first 72 hr. Both nuclei and microsomes showed a decrease in phosphatidylcholine (PC) content. Microsomes showed an increase in PC arachidonate while nuclei showed a decrease. Also, microsomes showed increased activity of phospholipase A_2 (PLA_2) while nuclei did not. These observations are consistent with the hypothesis that the absence of diene conjugates in liver microsomes in the rats on the CF diet may reflect the increased rate of removal of peroxidized fatty acids by phospholipase A_2.
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Rats fed a choline-free (CF) diet devoid of any added or known carcinogen, including aflatoxin B_1, develop liver cancer (1-3). The liver cells show a highly reproducible sequence of early biochemical and biological changes. Triglyceride accumulation begins at 8 hr and nuclear lipid peroxidation at 24 hr. Lipid peroxidation, as measured by the formation of diene conjugates (4), is seen in liver nuclei but not in liver microsomes 24 hr to 63 days on the dietary regimen (4,5). This is followed by DNA alterations, liver cell death, and liver cell proliferation by 5 days, and initiation of hepatocarcinogenesis by 10 wk (4-10).

It has been reported that the microsomes prepared from rats fed a CF diet show characteristic alterations in phospholipid composition (6). In view of the difference in observed appearance of diene conjugates in nuclei but not in microsomes (4,5) and the suggested relevance of nuclear lipid peroxidation to initiation of liver carcinogenesis (7), it became important to determine whether nuclei show the same changes as microsomes in their phospholipid content on feeding a CF diet. Also, because of the differential effect of phospholipase A_2 on the removal of peroxidized arachidonic acid from phospholipid (8), it was of interest to determine whether this enzyme shows different responses to a CF diet in nuclei and microsomes.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, MA), weighing 150 to 160 g, were used. The rats wre maintained on Purina Rodent Laboratory Chow (catalogue 5001) for 1 wk on a 12 hr day/night cycle. They were given food *ad libitum* and were acclimatized to their new environment for 1 wk before the start of the experiments.

Diet and chemicals. The choline-free (CF) and choline-supplemented (CS) diets were obtained from Dyets (Bethlehem, PA). The CF and CS diets were the same as described previously (2). Both diets contained 1800 mg L-methionine per kg, but only the CS diet had added choline (8.0 g choline chloride per kg diet). Diets were stored at 5°C, were analyzed regularly to detect potential contamination with aflatoxin B_1, nitrosamines and other potential mutagens (2), and were provided fresh to the rats on alternate days to minimize deterioration at room temperature.

1-Palmitoyl-2-[9′,10′(N)-^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine (sp. act., 58.0 Ci/mmole) was obtained from New England Nuclear (Dupont Canada Inc., Mississauga, Ontario, Canada). Phosphatidylcholine, phosphatidylethanolamine, butylated hydroxytoluene (BHT), Triton X-100, bovine serum albumin, calcium chloride and glycine were purchased from Sigma Chemical Co. (St. Louis, MO). Chloroform, methanol and n-hexane were purchased from Caledon Labs (Mississauga, Ontario, Canada).

Experimental design. Animals were divided into two groups of 12 animals each. One group was fed the CS diet while the other was given the CF diet after an overnight fast. A set of 3 animals each from both groups were sacrificed after 1, 2 or 3 days on the respective diets.

Preparation of subcellular organelles and assays. The livers were excised from the diethyl ether-anaesthetized rats, washed in ice-cold normal saline, and rapidly processed for the preparation of nuclei and microsomes. All subsequent steps were carried out at 4°C. Liver nuclei were isolated by the method of Blobel and Potter (9), with the addition of 1 mM EDTA (ethylenediaminetetraacetic acid) to the sucrose/TKM buffer. The purified nuclei were washed three additional times with 0.25 M sucrose/TKM buffer [5 mM Tris-HCl (pH 7.4 at 4°C), 2.5 mM KCl, 5.0 mM $MgCl_2$ and 0.24 M sucrose containing 1 mM EDTA] by sedimentation at 700 × *g* for 10 min and resuspension in 1 mL of the same buffer. The purity of the nuclei was monitored by light and electron microscopy as well as by biochemical markers assay as described (5). A 10% liver homogenate (w/v) in 0.25 M sucrose/TKM buffer, containing 1 mM EDTA (pH 7.4), was prepared for microsome separation. Homogenates were centrifuged at 10,000 × *g* for 10 min in a Sorval RC 5B centrifuge and then for 60 min in a Beckman ultracentrifuge (model L5-75, Beckman Instruments Inc., Palo Alto, CA) at 100,000 × *g* using a 60 Ti rotor to yield a microsomal pellet, which was then suspended in 1 mL of the TKM buffer.

Phospholipase A_2 activity in liver homogenate, nuclei and microsomes was assayed essentially by the method of Katsumata *et al.* (10). The constituents in the final 1-mL incubation mixture were 100 μmoles of glycine NaOH buffer, 2-3 mg of subcellular organelle protein, 2.5 μmoles of sodium deoxycholate, 2 μmoles of $CaCl_2$, 10 μg of bovine serum albumin and 150 nmoles of substrate. The mixture was incubated at 37°C for 1 hr and radioactive palmitic

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Abbreviations: CF, choline-free; CS, choline-supplemented; EDTA, ethylenediaminetetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2; TLC, thin-layer chromatography.

acid was extracted with 10 mL hexane containing 0.1% acetic acid in the presence of anhydrous sodium sulfate (0.5 g/mL). Activity of phospholipase A_2, as counts per minute per mg protein, was directly determined based on the radioactivity present in the hexane phase.

Lipids were extracted with chloroform/methanol (2:1, v/v) by the method of Folch *et al.* (11). Total phospholipids were quantitated by the method of Bartlett (12) as modified by Marinetti (13). Aliquots of the extracts were removed and chromatographed in duplicate along with standards for the separation and analysis of phospholipid classes by one-dimensional thin-layer chromatography (TLC) on Silica Gel G60, using the solvent system chloroform/methanol/7N ammonium hydroxide (230:90:15, v/v/v). Individual phospholipids were made visible under ultraviolet (UV) light after spraying the TLC plates with 1,6-diphenylhexatriene and were identified by comparison with co-chromatographed standards. The phospholipid bands were scraped from the plates and quantitated by phosphorus assay upon digestion in the presence of the gel. The fatty acid composition of phospholipid classes was measured upon methanolysis of the individual phospholipid classes for 12 hr in 4% H_2SO_4 in methanol at 90°C. The methyl esters were extracted with hexane.

Gas-liquid chromatography (GLC). GLC of the methyl esters was carried out on a Hewlett-Packard model 5880 (Mississauga, Ontario, Canada) automatic gas chromatograph using a level IV terminal and a 10 mM open granular glass capillary column (0.25 mm i.d.) with its wall coated with SP-2330 liquid phase (68% cyanopropyl and 32% phenylsiloxane) supplied by Supelco (Bellefonte, PA). Injector temperature was 270°C. Separations of fatty acid methyl esters were under temperature programming from 100 to 160 at 20°C/min and then at 2°C/min, or alternatively from 100 to 180°C at 20°C/min and then at 2°C/min, using hydrogen as carrier gas. The peaks were identified by reference to cochromatographed standards (14).

RESULTS

In the nuclear fraction (Table 1), the relative content of phosphatidylcholine as percentage of the total phospholipid, after 24, 48 and 72 hr on the CF diet, is decreased by 14 to 18%. Phosphatidylethanolamine (PE) shows a relative increase by 28 to 48%, while the phosphatidylcholine/phosphatidylethanolamine ratio is decreased by 33 to 43%. The total phospholipid content in each group remained the same over the 72-hr period.

In the microsomal fraction (Table 2), the pattern of decreasing phosphatidylcholine (PC), increasing PE and decreasing PC/PE is similar to that in the nucleus. Although the increase of PE at 24 hr is smaller than at 48 and 72 hr, it is not clear whether this is biologically significant in view of the small number of animals used. However, the values at all three time points and for each determination parallel very closely those seen in the nuclear fraction. The total phospholipid content in each group remained the same over the 72-hr period.

Associated with the changes in the quantities of PC and PE are alterations in the composition of some fatty acids. In the nuclear fraction (Fig. 1), the largest changes involve linoleic (18:2) and arachidonic (20:4) acids which show decreases of over 50% and about 40%, respectively, in the PC fraction and much smaller changes in PE. Several other fatty acids show small decreases or increases in both phospholipids.

The fatty acid compositions of PC and PE from microsomes show several differences (Fig. 2). The most striking is a large increase (about 40%) in arachidonic acid in the PC fraction. In contrast, there is a decrease in linoleic acid similar to that seen in the nuclei. In PC, 16:0, 16:1 and 18:0 decrease in the microsomes while the nuclear

TABLE 1

Nuclear Membrane Phosphatidylcholine and Phosphatidylethanolamine in the Liver of Rats Fed a CS or CF Diet

Time period	No. of animals	% of total phospholipid phosphorus		
		PC	PE	PC/PE
24 hr				
CS	3	62.0 ± 4.0	15.8 ± 1.6	3.9 ± 0.3
CF	3	52.7 ± 2.8	20.2 ± 1.4	2.6 ± 0.3[a]
48 hr				
CS	3	64.6 ± 0.14	18.3 ± 0.05	3.5 ± 0.01
CF	3	53.1 ± 0.33	27.1 ± 0.73[a]	2.0 ± 0.04
72 hr				
CS	3	60.4 ± 0.93	18.3 ± 0.14	3.3 ± 0.05
CF	3	51.8 ± 1.56	25.5 ± 1.14[a]	2.0 ± 0.03[a]

[a]$p<0.05$.

TABLE 2

Microsomal Phosphatidylcholine and Phosphatidylethanolamine in the Livers of Rats Fed a CS or CF Diet

Time period	No. of rats	% of total phospholipid phosphorus		
		PC	PE	PC/PE
24 hr				
CS	3	51.5 ± 1.02	26.7 ± 1.02	1.9 ± 0.006
CF	3	43.1 ± 0.90	28.7 ± 0.48	1.5 ± 0.015[a]
48 hr				
CS	3	55.9 ± 1.21	25.5 ± 1.57	2.2 ± 0.142
CF	3	42.6 ± 1.38	34.2 ± 0.73	1.2 ± 0.022[a]
72 hr				
CS	3	52.4 ± 2.48	26.3 ± 1.23	2.0 ± 0.01
CF	3	42.5 ± 1.35	32.7 ± 1.14	1.3 ± 0.003[a]

[a]$p<0.05$.

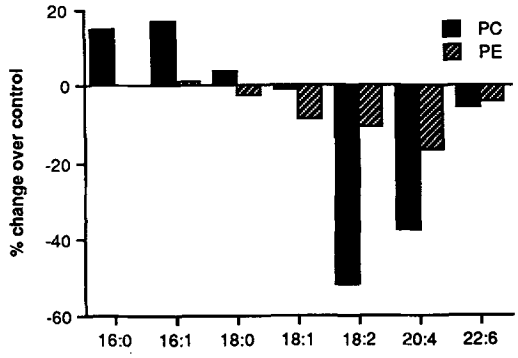

FIG. 1. Fatty acid composition of nuclear PC and PE from the livers of rats fed CF or CS (controls) diets for 72 hr.

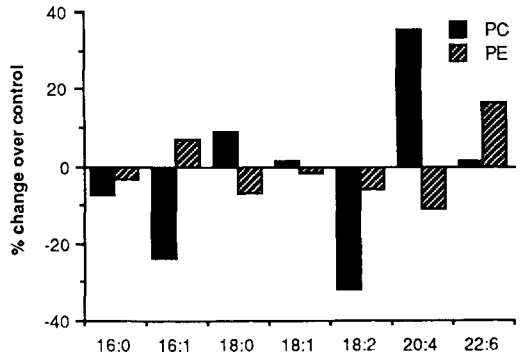

FIG. 2. Fatty acid composition of microsomal PC and PE from the livers of rats fed CF or CS (controls) diets for 72 hr.

TABLE 3

Phospholipase A_2 Activity in Whole Homogenate, Nuclei and Microsomes from Livers of Rats Fed CS or CF Diets

	Activity		
	Homogenate CPM/100 g liver	CPM/mg protein	
		Nuclei	Microsomes
72 hr			
CS	89152 ± 6353	736 ± 148	1379 ± 206
CF	133729 ± 840[a]	646 ± 56	1991 ± 229[a]

[a]$p<0.05$. Three animals in each group.

fraction shows increases. The variations in all the fatty acid determinations were well within ± 2 standard deviations.

Table 3 lists the activities of phospholipase A_2 (PLA_2) in the total liver homogenate and in the nuclear and microsomal fractions from animals fed the CS or the CF diets. The total homogenate shows a significant increase of about 50% and the microsomes of 34% in the animals fed the CF diet for 72 hr as compared to those on the CS diet. In contrast, the nuclear fraction shows a much lower activity as compared to the microsomes and whole homogenate and no significant difference between the values for the CF and CS animals at 72 hr.

DISCUSSION

The early changes observed in the phospholipid pattern (PC and PE) in the hepatic nuclear membrane of the rats on CF and CS diets are similar to those previously described for microsomes (6). However, the fatty acid patterns of PC and PE are quite different, especially in respect to the arachidonic acid content of microsomes and nuclei, as the latter show a decrease while the former show an increase. This may reflect different responses of PLA_2 in nuclei and microsomes to the feeding of a CF diet. The microsomes have twice the activity of the nuclei in the control animals and show a significant increase within 72 hr on the CF diet. A more rapid removal, especially of peroxidized arachidonic acid (8) from the microsomes and subsequent repair could account for observed differences between microsomes and nuclei in the generation of diene conjugates.

In the presence of peroxidized arachidonic acid in the phospholipid, the preferred target of PLA_2 is not arachidonate but its peroxidized form (8). In choline deficiency, peroxidized fatty acids could be removed by phospholipase A_2 and subsequently replaced by arachidonic acid in the microsomes. In the presence of a much lower level of activity of phospholipase A_2 in the nuclei, and without increase in activity in the animals fed the CF diet, the peroxidized arachidonic acid could remain in the nuclear membrane and thus become detectable as diene conjugates in this fraction.

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Levels of Thiobarbituric Acid Reactive Substances and the Cytocidal Potential of Gammalinolenic and Docosahexaenoic Acids on ZR-75-1 and CV-1 Cells

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To clarify the mechanism by which gammalinolenic acid (GLA) is more tumoricidal than docosahexaenoic acid (DHA), we have compared the incorporation of the respective exogenously added ethyl esters GLAe and DHAe into the phospholipids of tumorigenic ZR-75-1 and non-tumorigenic CV-1 cells relative to the ability of the cells to survive and to accumulate thiobarbituric acid reactive substances (TBARS). GLA and DHA were incorporated in the phospholipids to the same extent, but GLA disappeared more rapidly than DHA in both cell lines. GLAe induced about twice as much intracellular TBARS as DHAe in both cell lines, but killed ZR-75-1 cells four times more effectively than DHAe. DHAe induced 11–15 fmoles malondialdehyde-equivalents (MDA-eq)/cell in both ZR-75-1 and CV-1 cells, whereas GLAe induced 5–6 times more TBARS in ZR-75-1 cells (26–30 fmoles MDA-eq/cell) than in CV-1 cells (5–6 fmoles MDA-eq/cell). The results show that there is no difference in GLA and DHA incorporation into phospholipids, but that their metabolism differs in the two cell types. The data also suggest that the cytocidal potential is related to TBARS levels in a nonlinear fashion. The relationship between excess prostaglandin production and excessive cell death due to GLA is discussed.

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Previous studies have shown that gammalinolenic acid (GLA, 18:3n-6) kills tumorigenic cells at concentrations that are not cytocidal to non-tumorigenic cells (1,2). In contrast, docosahexaenoic acid (DHA, 22:6n-3) at the same concentrations affects tumorigenic and non-tumorigenic cells to a similar extent and kills significantly less cells than GLA (1,3). Using ZR-75-1 and CV-1 cells as model test systems, it was shown that GLA-induced cytotoxicity was associated with increased generation of oxygenated free radicals and with lipid peroxidation (4–6).

In cell free systems, the rate of fatty acid peroxidation is generally related to the number of double bonds. Since DHA (with 6 double bonds) would be more readily peroxidized than GLA (with three double bonds) (7,8), the difference observed between the cytocidal potential of GLA and DHA would suggest that the tumoricidal effects of GLA would only be related in part to autoxidation. It appears reasonable to assume that observed differences in tumoricidal effects also reflect differences in the uptake and the metabolic pathways between GLA and DHA. In the present study, we compare the incorporation of exogenously added DHAe and GLAe into the phospholipids of ZR-75-1 and CV-1 cells with the ability of the cells to survive and to accumulate TBARS.

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Abbreviations: DHA, docosahexaenoic acid; DHAe, docosahexaenoic acid ethyl ester; GLA, gammalinolenic acid; GLAe, gammalinolenic acid ethyl ester; MDA-eq, malondialdehyde-equivalent; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

MATERIALS AND METHODS

Materials. Ethyl esters of GLA (GLAe) and DHA (DHAe) (>99% pure) were purchased from NuChek Prep (Elysian, MN). 1,1,3,3-Tetramethoxypropane was obtained from Aldrich (Milwaukee, WI).

Fatty acids. Ethyl esters of GLA and DHA were dissolved in absolute ethanol and stored as stock solutions (10 mg/mL) under a nitrogen atmosphere at −75°C. For experiments, dilutions were freshly prepared from stock solutions using growth medium so that the final ethanol concentration equaled 0.2%.

Cells. Human breast (ZR-75-1) carcinoma cells and established normal simian kidney cells (CV-1) were obtained from the American Type Culture Collection. Stock cultures were grown in glass bottles in bicarbonate-buffered Dulbecco's modified Eagle medium containing 10% fetal calf serum, 2 mM glutamine, 50 μg gentamicin/mL and 2.5 μg fungizone/mL of growth medium. The same serum was used throughout; it contained less than 0.1 μg/mL of GLA and DHA.

Cell viability counts. Cells were seeded at 1×10^4 cells/well in 24-well tissue culture plates as previously described (1). One day after seeding, one set of cultures was supplemented with 20 μg/mL (*ca.* 60 μM) of GLAe or DHAe and incubated in 0.5 mL of growth medium at 37°C in a 5% CO_2 humidified incubator without medium change for the duration of the experiment. Unsupplemented control cultures received 0.2% final concentration of ethanol and were incubated in parallel. The concentration of added ethanol in the control cultures was equivalent to the concentration of ethanol present in supplemented cultures. Supplemented and unsupplemented cultures were examined in parallel after identical incubation periods. Cell viability counts were determined by trypan blue exclusion in a Neubauer hemacytometer. Before counting, the cells were detached following treatment with trypsin-EDTA solution, pooled, and centrifuged in their respective culture medium. The percentage of dead cells was evaluated as the number of stained cells × 100/total number of cells.

Intracellular TBARS levels. Cultures were made from 5×10^4 cells seeded in 35-mm culture dishes. One day after seeding, the medium was removed and the cultures were supplemented with 20 μg/mL of GLAe or DHAe, or kept unsupplemented, and incubated in 2 mL of growth

medium as described above. At the indicated times, cells were detached by trypsinization, washed in PBS, and assayed for TBARS according to the method of Gavino *et al.* (9). Briefly, 1 mL of 20% trichloroacetic acid was added to 2 mL of cells resuspended in PBS, pH 7.0. Two mL of 0.67% TBA was added. This mixture was incubated for 20 min at 90°C and centrifuged at 1200 × *g* for 10 min at 4°C. The absorbance of the supernatant was measured at 532 nm using PBS as a reference. Absorbance was converted to pmoles MDA-eq from a standard curve using 1,1,3,3-tetramethoxypropane as the source of malondialdehyde. All experiments were done at least in triplicate.

TBARS levels in cell-free cultures. Two mL of growth medium in 35-mm culture dishes were supplemented with 20 μg/mL of GLAe or DHAe and incubated as described above. Culture dishes containing growth medium (but without GLAe or DHAe supplementation) were incubated in parallel under otherwise similar conditions. At the indicated time points, the medium was assayed for TBARS according to the method of Gavino *et al.* (9) as described above. Growth medium was used as a reference. All experiments were done in triplicate.

Phospholipid fatty acids. Cell cultures seeded with 6 × 10^6 cells per 243 × 243 × 18 mm Bio-Assay dish (Nunc) were challenged with GLAe or DHAe, as described above. At 1, 3 and 7 days after supplementation, the cells were trypsinized and washed three times with PBS, pH 7.0. Lipids were extracted from cell pellets which had been resuspended in 0.1 volume of PBS with chloroform/methanol (2:1, v/v) containing 0.02% butylated hydroxytoluene (BHT) in 16 × 125 mm tubes. After centrifugation, the lower phase was taken to dryness and redissolved in 100 μL of high-performance liquid chromatography (HPLC) grade chloroform. The total phospholipid fraction was separated by thin-layer chromatography using a solvent system consisting of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Methyl esters were prepared by treatment of samples with 14% boron trifluoride in methanol and the relative fatty acid composition was determined by gas-liquid chromatography according to Manku *et al.* (10).

Statistical analysis. The results are expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to test significant variations between the fatty acid treatments. Statistical significance was analyzed by two-tailed Student's *t*-test.

RESULTS AND DISCUSSION

Table 1 shows that both ZR-75-1 and CV-1 cells had incorporated GLA and DHA into their phospholipids to the same extent at day 1 after supplementation. The levels of GLA were maximal in both ZR-75-1 and CV-1 cells after the first day of supplementation and decreased significantly thereafter. In contrast, the proportion of DHA was maximal at day 3 and decreased thereafter in both cell lines at a slower rate than was seen for GLA. After day 1, the proportion of DHA remained 2–10 times higher than the levels of GLA in both cell lines.

Table 2 demonstrates that more TBARS were found in cells supplemented with the fatty acids than in unsup-

TABLE 1

Time Course Levels[a] of GLA and DHA in the Phospholipids of ZR-75-1 and CV-1 Cells

		GLA		DHA	
Cells	Day	Untreated	Treated	Untreated	Treated
ZR-75-1	1	<0.1	9.2 ± 1.3[b]	2.1 ± 0.1	8.3 ± 0.5
	3	<0.1	4.9 ± 0.9	3.0 ± 0.1	12.4 ± 0.8
	7	<0.1	2.7 ± 0.5	2.0 ± 0.4	8.0 ± 0.2
CV-1	1	<0.1	11.0 ± 1.6	2.8 ± 0.1	8.6 ± 0.3
	3	<0.1	3.5 ± 0.8	3.9 ± 0.1	16.3 ± 0.1
	7	<0.1	1.1 ± 0.2	3.4 ± 0.2	12.3 ± 0.2

[a]Weight % of total fatty acids.

[b]Mean ± SD of three samples.

TABLE 2

TBARS Levels and Viability of ZR-75-1 and CV-1 Cells Exposed to GLAe and DHAe

		fmoles MDA-Eq/cell[a]			% Dead cells		
Cell	Day	Control	GLAe	DHAe	Control	GLAe	DHAe
ZR-75-1	3	0.3 ± 0.3[b]	28.0 ± 4.6	15.0 ± 2.0	14 ± 5	15 ± 5	12 ± 3
	4	0.8 ± 0.5[b]	30.0 ± 3.2	14.0 ± 1.8	13 ± 8[b]	52 ± 8[c]	18 ± 6
	7	0.2 ± 0.3[b]	26.5 ± 2.7	11.3 ± 3.9	17 ± 3[b]	99 ± 2[c]	25 ± 7
CV-1	3	2.1 ± 3.5	5.6 ± 2.8[c]	12.3 ± 3.7	12 ± 3	13 ± 4	14 ± 5
	4	0.0 ± 0.5[b]	5.4 ± 1.7[c]	12.1 ± 1.0	5 ± 2	6 ± 3	7 ± 2
	7	0.5 ± 0.3[b]	6.2 ± 1.1[c]	11.4 ± 3.0	13 ± 5	15 ± 5	20 ± 7

[a]Mean ± SD of three samples.

[b]ANOVA between fatty acid treatments at $p>0.01$.

[c]Significant difference of the mean (two-tailed Student's *t*-test) between ZR-75-1 and CV-1 cells on indicated day at $p>0.01$.

TABLE 3

TBARS Values in Cell-Free Culture Medium Supplemented with GLAe and DHAe[a]

Day	pmoles MDA/2mL medium ($\times 10^2$)[b]		
	Control	GLAe	DHAe
1	6.3 ± 6.0	10.7 ± 6.7	59.5 ± 4.8[c]
3	14.3 ± 3.4	17.4 ± 2.2	49.0 ± 7.0[c]
4	30.8 ± 2.7	28.5 ± 5.5	70.9 ± 8.3[c]
7	4.5 ± 6.3	7.2 ± 7.5	66.4 ± 3.4[c]

[a]The medium contained 10% fetal calf serum.

[b]Mean ± SD of three samples.

[c]Significant difference between DHAe and GLAe by two-tailed Student's *t*-test at $p>0.01$.

plemented cells. GLAe induced about 5–6 times more TBARS in ZR-75-1 cells than in CV-1 cells. In contrast, the TBARS levels found in ZR-75-1 and CV-1 cells exposed to DHAe were the same and reached about half the TBARS levels found in GLAe-supplemented ZR-75-1 cells. Conversely, DHAe as compared to GLAe generated the greatest accumulation of TBARS in a cell-free system (Table 3).

The percentage of dead cells were highest in ZR-75-1 cells treated with GLAe. This cytocidal effect corresponded to the higher TBARS levels in ZR-75-1 cells as compared to those in CV-1 cells. Lower TBARS levels with DHA corresponded to lower cytocidal activity in relation to GLA. However, higher TBARS with DHA-treated cells did not affect cytotoxicity relative to untreated controls.

The data show that there is no difference in GLA and DHA incorporation into phospholipids, but that their metabolism differs in the two cell types and that the cytocidal potential relates nonlinearly to TBARS levels. The nature of the TBARS has not been established here but it is plausible that hydroperoxide and endoperoxide degradation products account chiefly for the TBARS levels detected in the fatty acid-supplemented cells. It is well recognized that both GLA and DHA produce hydroperoxides by nonenzymatic mechanisms, such as autoxidation processes, but GLA can also be metabolized to dihomogammalinolenic acid and, to some extent, to arachidonic acid, both of which enzymatically give rise to prostaglandins, whereas DHA is a potent inhibitor of prostaglandin synthesis (11). Thus, the ability of GLA to produce endoperoxide and endoperoxide breakdown products suggests that the difference observed in GLA and TBARS levels might be associated with prostaglandin synthesis and that they may contribute to the cytocidal effects. Another possibility is that DHA could influence the availability of arachidonic acid for endoperoxides (12). Although plausible, this hypothesis is less likely because previous studies have demonstrated that eicosanoid synthesis is not involved in the cytocidal effects of GLA (4,13). Thus, a different radical-driven process developing in parallel or concurrently to prostaglandin synthesis and/or autoxidation may be operative (4,5). Whether or not this relates to the gradual accumulation of conjugated trienes, which has been reported to be associated with lethal treatments, remains to be determined (14). The results substantiate the hypothesis that n-6 and n-3 polyunsaturated fatty acids induce cytotoxic effects by differing mechanisms (13,15).

To differentiate between these possibilities, and because of the uncertainties in comparing tissues of different origins, the use of genetically-related tumorigenic and non-tumorigenic cells would be desirable. Using a series of closely-related tumorigenic and non-tumorigenic cell lines, we have confirmed that tumorigenic cell lines were more sensitive than corresponding non-tumorigenic cell variants to GLA challenge (2,16). Hence, these cell lines should prove suitable for comparing possible mechanisms of the cytocidal mode of action of GLA, DHA and other n-6 and n-3 polyunsaturated fatty acids.

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Phospholipid Composition of Cultured Human Endothelial Cells

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Detailed analyses of the phospholipid compositions of cultured human endothelial cells are reported here. No significant differences were found between the phospholipid compositions of cells from human artery, saphenous and umbilical vein. However, due to the small sample sizes, relatively large standard deviations for some of the phospholipid classes were observed. A representative composition of endothelial cells is: phosphatidylcholine 36.6%, choline plasmalogen 3.7%, phosphatidylethanolamine 10.2%, ethanolamine plasmalogen 7.6%, sphingomyelin 10.8%, phosphatidylserine 7.1%, lysophosphatidylcholine 7.5%, phosphatidylinositol 3.1%, lysophosphatidylethanolamine 3.6%, phosphatidylinositol 4,5-bisphosphate 1.8%, phosphatidic acid 1.9%, phosphatidylinositol 4-phosphate 1.5%, and cardiolipin 1.9%. The cells possess high choline plasmalogen and lysophosphatidylethanolamine contents. The other phospholipids are within the normal biological ranges expected. Phospholipids were separated by high-performance liquid chromatography and quantified by lipid phosphorus assay.
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Phospholipids, and particularly the polyphosphoinositides, play an important role in endothelial cell receptor function. Stimulation of the bradykinin receptor has been shown to affect polyphosphoinositide turnover in endothelial cells (1,2). This turnover is mediated by the combined action of phospholipase C and diacylglycerol lipase on the polyphosphoinositides, thereby releasing arachidonic acid (3,4). Several alternative pathways have been proposed for arachidonic acid release in endothelial cells. These include the action of phospholipase A_2 (5,6) and a combined calcium independent phospholipase A_1 and lysophospholipase pathway (4). In other cell systems, decreases in choline plasmalogen have been linked to bradykinin receptor activation (7).

Plasmalogen hydrolysis has been implicated in several receptor mechanisms which result in release of arachidonic acid (8). Phosphatidylcholine (PtdCho) is a major contributor of arachidonic acid following thrombin stimulation (6) or stimulation by the calcium ionophore A23187 (9). These decreases in PtdCho may be due to the hydrolysis of choline plasmalogen (7,8) or the receptor mediated breakdown of PtdCho (10,11). Regardless, these receptor mechanisms all involve the degradation of membrane phospholipids resulting in the release of arachidonic acid for eicosanoid formation (12-17).

Partial phospholipid compositions of endothelial cells have been reported for different species (5,18-25). However, in order to better understand the role of phospholipids in receptor function, the complete and detailed phospholipid composition of endothelial cells needs to be known. In the present study, the phospholipid compositions of human endothelial cells from three vascular sources were compared in order to determine whether phospholipid composition is dependent upon the vascular source. This report is the first to describe the simultaneous extraction and high-performance liquid chromatography (HPLC) separation of plasmalogens and polyphosphoinositides from cultured cells.

MATERIALS AND METHODS

Three vascular sources were used to establish separate human endothelial cell cultures. Cells were isolated from cadaver aorta artery, from bypass patient's saphenous vein, and from newborn umbilical veins by treating the luminal surface with 0.1% type II collagenase (26). The cells were plated on human fibronectin coated glass tissue culture flask (23 cm^2) containing M-199 media Gibco (Gaithersburg, MD) supplemented with 10% fetal bovine serum, 150 μg/mL endothelial cell growth factor (27), 90 μg/mL Na-heparin (28), and antibiotics. The cells in a flask upon reaching confluency were split and plated on 100-mm glass cell culture plates. Cells were used from passage 5 through 12. Their endothelial origin was confirmed by the presence of factor VIII antigen (29).

Confluent cells were extracted using n-hexane/2-propanol (3:2, v/v) (30). The use of this extraction method avoids the use of toxic solvents, decreases the amount of extractable protein, and avoids the loss of water-soluble phospholipids. Prior to extraction the medium was removed and the cells were washed with two 3-mL portions of cold PBS buffer to remove traces of medium. The plates were immediately placed upon dry ice prior to extraction and frozen to minimize membrane damage and activation of acylhydrolases during extraction (31). Two 3-mL aliquots of n-hexane/2-propanol (3:2, v/v) were used to extract the lipids from the cells. The initial aliquot was added to the frozen cells which were subsequently removed from the plate by using a Teflon cell scraper. The second aliquot was used to rinse the plate. Most cellular debris was removed by filtration through glass wool. The extracts were kept under N_2 to minimize auto-oxidation of lipids.

Extracts were stored at −20°C. Prior to phospholipid separation, the cells were subjected to ultrafiltration using a Rainin 0.2 μm Nylon filter (Woburn, MA). The sample was dried under nitrogen and redissolved in a known

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Abbreviations: Cer*P*Cho, sphingomyelin; Gpl, glycerophospholipid; HAE, human arterial endothelial; HPLC, high-performance liquid chromatography; HSVE, human saphenous vein endothelial; HUVE, human umbilical vein endothelial; lysoPtdCho, lysophosphatidylcholine; lysoPtdEtn, lysophosphatidylethanolamine; PlsCho, choline plasmalogen; PtdCho, phosphatidylcholine; PlsEtn, ethanolamine plasmalogen; PtdEtn, phosphatidylethanolamine; Ptd_2Gro, cardiolipin; PtdIns, phosphatidylinositol; PtdIns4*P* phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; TLC, thin-layer chromatography.

volume of n-hexane/2-propanol/water (3:2, + 5.5% by volume) prior to high-performance liquid chromatography (HPLC).

The cell extracts were separated into major phospholipid classes by HPLC. Solvents used were HPLC grade n-hexane and 2-propanol from E.M. Science (Cherry Hill, NJ). Solvents were filtered through a 0.5-μm Millipore FH-type Nylon filter (Bedford, MA) and degassed. Solvent A was n-hexane/2-propanol (3:2, v/v), solvent B was n-hexane/2-propanol/water (3:2 + 5.5% by volume). Water was purified using a Millipore water purification system. The HPLC instrument consisted of two Altex (Berkeley, CA) 100A pumps, an Altex 420/421 controller and an Altex model 210 injection port. The Dupont Zorbax Silica column (4.6 mm × 250 mm 5–6 μm; Wilmington, DE) was maintained at a constant temperature of 34°C using a Jones chromatography heating block (Columbus, OH). An ISCO (Lincoln, NE) V4 ultraviolet (UV) variable wave length detector was used to detect peaks at 205 nm.

The chromatographic procedure (32) permitted the separation of the major phospholipids including the separation of the acidic phospholipids phosphatidylserine (PtdSer) and phosphatidylinositol (PtdIns) as well as the resolution of lysophosphatidylethanolamine (lysoPtdEtn). The polyphosphoinositides were also separated (33). Retention times for each phospholipid class were according to polarity with the elution order being from least polar to most polar. The lower limit of sensitivity was 100 nmol of injected lipid phosphorus.

Plasmalogens were separated by HPLC after acidic hydrolysis (34). The ethanolamine glycerophospholipid (EtnGpl) and choline glycerophospholipid (ChoGpl) peaks were collected, dried under N_2, and then inverted over 5 drops of concentrated hydrochloric acid in a test tube cap for two minutes. This caused hydrolysis of the alkenyl ether bond of the plasmalogens while the alkylacyl and diacyl bonds remained intact. The sample was reextracted and rechromatographed utilizing the same separation procedure as used in the initial separation.

The alkylacyl and diacyl fractions were then collected and subjected to alkaline hydrolysis in 2 mL of 0.1 M NaOH in methanol. After 15 min the reaction was terminated by addition of 1 mL of ethyl formate (35). Cleavage of the alkali-labile ester linkage caused formation of glycerophosphocholine or glycerophosphoethanolamine and of alkylglycerophosphocholine or alkylglycerophosphoethanolamine. The products were separated following extraction of the methanol phase with chloroform/isobutanol/water (4:2:3, v/v/v) followed by centrifugation to facilitate phase separation. The top layer contained glycerophosphocholine or glycerophosphoethanolamine, the bottom layer contained the ether lysophospholipids.

Phospholipid classes were quantitated by measuring lipid phosphorus (36).

RESULTS

The compositions of human saphenous vein (HSVE), human umbilical vein (HUVE) and human arterial endothelial cells (HAE) are reported in Table 1. LysoPtdEtn represented an abnormally high mole percentage of the total lipid phosphorus in the cells from all sources. The polyphosphoinositide values are also reported. No statistically significant differences in the proportions of phospholipid classes were found between the various sources of endothelial cells. The most important finding is the high lysoPtdEtn level seen in all three sources.

Because no differences were found between the major phospholipids from various sources, the plasmalogen content of only the HUVE cells was determined. The PlsEtn content was 40.5% of the EtnGpl and the PlsCho content was 11.6% of ChoGpl. These values represent 7.1% and 5.0% respectively of the total lipid phosphorus. The compositions of the choline and ethanolamine glycerophospholipid subclasses are listed in Table 2.

The amount of lipid phosphorus injected onto the column was at or below the desirable lower limit of sensitivity (100 nmol) due to the small sample sizes available from

TABLE 1

Phospholipid Composition of Cultured Human Endothelial Cells[a]

	HUVE n = 12 × S.D.	HSVE n = 10 × S.D.	HAE n = 12 × S.D.
Ptd_2Gro	2.60 ± 2.88	2.06 ± 1.05	1.08 ± 0.85
PtdEtn+	17.63 ± 1.98	16.86 ± 3.11	19.06 ± 3.78
PtdOH	2.37 ± 3.09	1.81 ± 1.64	1.56 ± 0.76
PtdIns	1.90 ± 3.09	2.99 ± 2.21	4.26 ± 2.31
lysoPtdEtn	3.12 ± 3.36	5.26 ± 2.22	2.54 ± 2.07
PtdSer	5.36 ± 2.70	8.86 ± 1.76	7.15 ± 2.87
PtdCho+	42.76 ± 6.26	39.03 ± 6.56	39.12 ± 3.92
Cer*P*Cho	8.91 ± 2.69	10.52 ± 2.39	12.96 ± 5.04
PtdIns4P	1.56 ± 1.75	1.19 ± 0.94	1.90 ± 1.83
lysoPtdCho	7.53 ± 5.04	8.07 ± 4.68	7.02 ± 4.84
$PtdIns4,5P_2$	1.24 ± 0.94	1.99 ± 1.05	2.09 ± 1.26

[a]Values are mole % of total phospholipid. Tukey's multiple comparison test was used for statistical evaluation. +PtdEtn and PtdCho represent the sum of alkenylacyl, alkylacyl and diacyl fractions. Abbreviations are: Cer*P*Cho, sphingomyelin; HAE, human arterial endothelial; HSVE, human saphenous vein endothelial; HUVE, human umbilical vein endothelial; lysoPtdCho, lysophosphatidylcholine; lysoPtdEtn, lysophosphatidylethanolamine; PlsCho, choline plasmalogen; PtdCho, phosphatidylcholine; PlsEtn, ethanolamine plasmalogen; PtdEtn, phosphatidylethanolamine; Ptd_2Gro, cardiolipin; PtdIns, phosphatidylinositol; PtdIns4*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdOH phosphatidic acid; PtdSer phosphatidylserine.

TABLE 2

Composition of Ethanolamine and Choline Glycerophospholipid Subclasses[a]

	EtnGpl class (%)	Total Gpl (%)
Diacyl	45.2 ± 1.4	8.0
Alkenylacyl	40.5 ± 1.9	7.1
Alkylacyl	14.3 ± 0.5	2.5
	ChoGpl class (%)	**Total Gpl (%)**
Diacyl	78.4 ± 1.1	33.5
Alkenylacyl	11.6 ± 0.9	5.0
Alkylacyl	10.0 ± 1.6	4.3

[a]The values are means ± standard deviations. For EtnGpl and ChoGpl, n = 4. Abbreviations are: EtnGpl, ethanolamine glycerophospholipid; ChoGpl, choline glycerophospholipid; and Gpl, glycerophospholipid.

the cultures. Thus, the variability between samples increases especially for phospholipid classes that represent a small percentage of total lipid phosphorus.

DISCUSSION

The purpose of this study was to determine the phospholipid composition of human endothelial cells in culture and the dependence of this composition on the vascular source. The separation of phospholipids by HPLC allows for good recovery of all phospholipids, improved phosphorus determination and detection of smaller changes in phospholipid composition with statistical significance due to a lower variance than based on thin-layer chromatography (TLC) (37). The extraction procedure used permits a good recovery of phospholipids while minimizing the amount of protein in the extract. The procedure increases polyphosphoinositide recovery and avoids the need for a two-phase system which can cause losses of lysoglycerophospholipids (30). Freezing the cells prior to extraction decreases the mechanical trauma incurred by the cells during their removal from the plates (31). Hence, our values should represent more closely the actual composition of the cells in culture.

Several papers have reported partial phospholipid compositions of endothelial cells from various vascular sources and species (5,19–25). A composition of 25.3% PtdEtn, 36.3% PtdCho and 12.8% Cer*P*Cho in HUVE cells has been reported by Rastogi and Nordøy (19), which is similar to our data. A plasmalogen content in HUVE cells of 42.5% PlsEtn in EtnGpl and 6.8% PlsCho in ChoGpl has been reported by Blank *et al.* (22). However, their values for the choline glycerophospholipid diacyl (88.6%) and alkylacyl (4.6%) subclasses differ from our values of 78.4% and 10%, respectively. The reported values for the two ethanolamine glycerophospholipid subclasses were 52.6% for the diacyl and 4.9% for the alkylacyl fractions (22). Our values were 45.3% and 14.3% for the diacyl and alkylacyl subclasses, respectively. These differences may reflect the different methodologies used to quantitate the EtnGpl and ChoGpl subclasses.

Values for the proportions of choline and ethanolamine glycerophospholipid subclasses reported by Takamura *et al.* (25) also differ from our values. The differences could be due to the different methodology used to separate the subclasses. The reported phospholipid compositions for both umbilical vein and arterial endothelial cells (25) are also considerably different from our values. Phospholipid separations by TLC usually have lower recoveries than HPLC separations (our recoveries are 98.3% ± 3), and TLC may not completely resolve all classes of phospholipids because of the lower number of theoretical plates.

Values for PtdEtn and PtdCho composition from rat and human brain endothelial cells (24) compare closely to our values, although a difference exists in Cer*P*Cho contents with 20.4% and 17.0% for rat and human brain, respectively, compared with our value of 10.8%. The difference may be due the fact that the cells were freshly isolated, and not cultured. Isolated rat brain endothelial cells have 46.3% PlsEtn in EtnGpl (18), which is close to our value. The polyphosphoinositide content of endothelial cells has not been reported previously for any species. Guinea pig brain contains 0.58% PtdIns4*P* and 2.58% PtdIns (4,5)P_2 (38). Our values are within this range. Our data compare favorably to several literature values for the partial phospholipid composition of endothelial cells from various species and vascular sources and are the first to include polyphosphoinositides.

The cells exhibited a high lysoPtdEtn and a relatively high lysoPtdCho content. The position of the fatty acid was not determined. Regardless of fatty acid position, these lysophospholipids are known to have detergent properties on cellular membranes (39). High concentrations of endogenous lysophospholipids are generally assumed to increase membrane fluidity and to give rise to subtle changes in cellular surface. The high levels in human endothelial cells may be related to the apparent ease of thrombosis (40). Increases in membrane fluidity may also cause the cell membrane to be more susceptible to mechanical damage accounting for the observed morphological changes such as crater formation in endothelial cells (41). High levels of lysolipid may modulate the effect of injury and interaction of the endothelium with various hormones (42) because lysophospholipids can alter the functioning of membrane-bound enzymes (39,43).

Endothelial cells play a major role in vascular homeostasis (40). In order to understand the lipid metabolism of endothelial cells, the basic phospholipid composition must be known. It has been hypothesized that lipid composition varies with vascular source based on observations of ultrastructural differences depending on vessel size and location (19). In this study no dependence of phospholipid composition on vascular source was found. However, it should be noted that in some phospholipid classes the standard deviations were high. This is especially true for phospholipids which make up a small percentage of the total phospholipids. Differences in phospholipid classes molecular species dependent upon vascular source were not ruled out.

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Unusual Tetraene Sterols in Some Phytoplankton

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Sterols were analyzed from four phytoplankton strains which are under investigation as possible sources of food for oysters in culture. One strain of *Pyramimonas* contained only 24-methylenecholesterol as a major sterol component. *Pyramimonas grossii, Chlorella autotrophica* and *Dunaliella tertiolecta* each contained a complex mixture of C_{28} and C_{29} sterols with Δ^7, $\Delta^{5,7}$ and $\Delta^{5,7,9(11)}$ nuclear double bond systems. Sterols were found both with and without the C-22 side chain double bond. Ergosterol and 7-dehydroporiferasterol were the principal sterols in each of the latter three species, which also contained the rare tetraene sterols, 24-methylcholesta-5,7,9(11),22-tetraen-3β-ol and 24-ethylcholesta-5,7,9(11),22-tetraen-3β-ol.
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Most marine invertebrates examined have a complex sterol composition attributed to lack of sterol synthesis in the animal and incorporation of a wide variety of dietary sterols into the animal tissue (1). The oyster (*Crassostrea virginica*) has been shown to contain over forty sterols (2–4), but it cannot synthesize any of them from acetate (5–7). Recent research shows a strong correlation between oyster growth rate and the type and amount of dietary sterol present (8).

Approximately 60% of total oyster sterol is cholesterol, 24-methylenecholesterol and 24-methyl-22-dehydrocholesterol (2). The latter two sterols are found in specific diatoms (9) occurring in the estuarine environments, and several diatoms are known to provide a good food source for oysters (10). The source of the oyster's cholesterol has not been identified and most phytoplankton do not contain more than trace quantities of it (9).

The work described here is part of an effort to improve our knowledge of the effect of dietary sterol on oyster growth rates by examining a wide range of phytoplankton with varied sterol composition which are within the size range utilized by the oyster.

MATERIALS AND METHODS

Axenically cultured algae (strains 580, De, and 78) obtained from the Milford microalgal culture collection were prepared for analysis as previously described (11). Pyram 2 was a gift from Jeff Johanson of the Solar Energy Research Institute (Golden, CO). Dry algal samples (0.3–1.0 g) were extracted with $CHCl_3$/MeOH and sterols were isolated from the non-saponifiable lipid (11). Sterols were analyzed on a 30 m × 0.25 μm fused silica capillary column with a 0.25 mm film of SPB-1 (Supelco, Bellefonte, PA) at 255°C in a Varian Model 3500 chromatograph equipped with a Varian Model 8100 autosampler, an on-column injector, and a hydrogen flame detector (Varian Associates, Palo Alto, CA). Each unknown was injected alone and with a cholesterol standard for relative retention time (RRT) determination. Sterol identity was confirmed by electron impact gas chromatography/mass spectrometry (GC/MS) with a 30 m × 0.32 mm i.d. fused silica capillary column with a 0.25 mm film of DB-1.

RESULTS AND DISCUSSION

Pyramimonas sp. had a simple sterol composition of 99% 24-methylenecholesterol and 1% cholesterol (Table 1). However, the compositions of *Pyramimonas grossii, Chlorella autotrophica* and *Dunaliella tertiolecta* were complex. The principal sterols of *D. tertiolecta* and *C. autotrophica* have been identified previously as ergosterol and 7-dehydroporiferasterol (7,12), but detailed sterol analyses were not reported in either case. In *D. tertiolecta* the side chain methyl and ethyl of these compounds were determined by ^{13}C nuclear magnetic resonance spectrometry to be oriented in the β position (12). Side chain stereochemistry of *C. autotrophica* was not determined, but in other *Chlorella* species where it was determined, the C-24 methyl and ethyl groups were also β-oriented (9,13).

In this work, capillary gas chromatography confirmed the presence of major peaks for the above two sterols but

TABLE 1

Abundance of Sterols in Four Cultured Algae[a]

Sterol	RRT	Species[b] 1	2	3	4
Cholesterol	1.00	—	—	—	1
24-Methylenecholesterol	1.20	—	—	—	99
9(11)-Dehydroergosterol	1.08	3	1	1	—
Ergosterol	1.18	36	22	31	—
5-Dihydroergosterol	1.23	1	5	3	—
Ergost-5,7-dienol	1.35	3	—	—	—
Ergost-7-enol	1.41	20	8	4	—
Stigmasta-5,7,9(11),22-tetraenol	1.33	3	2	2	—
7-Dehydroporiferasterol	1.46	23	46	53	—
Stigmasta-7,22-dienol	1.51	1	9	4	—
Stigmasta-5,7-dienol	1.66	1	—	—	—
Stigmast-7-enol	1.71	6	4	1	—

[a]Abundances are expressed as % of total sterol. RRT are relative to cholesterol. C-24 orientation was not determined for any compound.

[b]Cultures were obtained from the Milford Culture Collection (strain No. in parentheses) except for *Pyramimonas* sp., which was from the Solar Energy Research Center.

1, *Chlorella autotrophica* (580); 2, *Dunaliella tertiolecta* (De); 3, *Pyramimonas grossi* (78); 4, *Pyramimonas* sp. (Pyram 2).

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Abbreviations: GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; RRT, relative retention time.

also detected the presence of a number of additional compounds. GC/MS enabled the identification of 7(8)-monoene sterols and 7,22-diene sterols with 28 and 29 carbon atoms (Table 1). In each case mass spectra were identical with those of reference compounds isolated from *Chlorella emersonii* (14). A small amount of 14α-methyl-5α-stigmast-9(11)-enol was identified in *Chlorella autotrophica* by comparison of its mass spectra with those reported by the Akihisa group (15,16). The 24α-epimer of this sterol (all sterols of *Chlorella* to date are 24β) has been reported in *Cucumis sativa* (15), and 14-methyl-5α-ergost-9(11)-enol has been reported recently in *Chlorella vulgaris* (16). Also detected were C_{28} and C_{29} tetraene sterols with GC retention times shorter than for the corresponding trienols ergosterol and 7-dehydroporiferasterol.

Tetraene sterols are not common, but a number of them are known to occur in living organisms. Cholesta-5,7,22,24-tetraenol has been isolated from *Tribolium confusum*, where it is an intermediate in the dealkylation of stigmasterol to cholesterol (17). Known tetraethenoid C_{28} sterols include 14-dehydroergosterol from a strain of *Aspergillus niger* (18), 24-dehydroergosterol from yeast (19), 25(27)-dehydroergosterol (protothecasterol) from the colorless alga, *Prototheca* (20), and 9(11)-dehydroergosterol from *Giberella fujikuroi*, *Candida lipolytica* and *Chlorella vulgaris* (16,21,22). The tetraene sterol reported here from *Chlorella*, *Dunaliella* and *Pyramimonas* has gas chromatographic characteristics (Table 1) and mass spectra identical to those of a synthetic sample of 9(11)-dehydroergosterol provided by Nes (20). Major mass spectra peaks were observed for the free sterol at *m/z* (rel. abundance), 394(8), 376(17), 361(3), 251(100) and 69(100).

Each of the algae containing 9(11)-dehydroergosterol also contained a C_{29} tetraene. Gas chromatographic retention times were 1.23 times those of the C_{28} tetraene in each case (Table 1), which is the increase expected for the addition of an extra carbon at C-28. Mass spectra of the tetraene from each alga were identical and were very similar to those of the C_{28} tetraene, especially with respect to the dominant base peak at *m/z* 251. This compound is identified as 24-ethylcholesta-5,7,9(11),22-tetraenol, a compound to our knowledge not previously reported to occur in nature. Species previously shown to produce C_{28} tetraenes did not produce C_{29} sterols (16,18–22). *Chlorella autotrophica*, *D. tertiolecta* and *P. grossii* each produces a mixture of C_{28} and C_{29} monoenes, dienes, trienes and tetraenes.

Gibberella produced 9(11)-dehydroergosterol only in stationary phase cultures (19). It should be pointed out that the cultures examined here also were harvested from the stationary phase. Detailed analyses from log phase cultures of these algae are not available. Such data may be important in the culture of algae for oyster rearing, because recent data from our laboratories indicate a correlation between algal sterol composition and the growth rates of oysters fed unialgal diets (8).

Neither the metabolic origin nor the role of 9(11)-dehydroergosterol is known. Its accumulation in stationary phase cultures prompts speculation of a new pathway *via* parkeol (lanosta-9(11),24-dien-3β-ol) or alternately by the dehydrogenation of ergosterol. The sterol composition of *D. tertiolecta* determined here is in agreement with that reported in previous work (12). It should be noted that while two other *Dunaliella* species have been reported to contain principally $\Delta^{5,7}$-sterols (23,24), *D. minuta* has 24-methylenecholesterol and 24-methylcholesterol as principal sterols (25), and *D. acidophila* contains 24-ethylcholesterol and 24-ethylidenecholesterol as its major sterols (26). An unialgal diet of *D. tertiolecta* supports moderate growth of oysters (27). Because sterol composition varies widely from species to species, the comparative nutritional value of these species is of great interest.

In our studies, oysters grew more poorly on *P. grossii* than on *D. tertiolecta* (G.H. Wikfors, unpublished data). We have no nutritional data on *Pyramimonas* sp. It is interesting to note that (as is the case with *Dunaliella*), sterol composition varies widely with species in *Pyramimonas*. In contrast with the species of *Pyramimonas* studied here, Volkman (28) has found isofucosterol to be the principal sterol of *Pyramimonas gelidicola*. In many phytoplankton taxa, names and taxonomic affinities of species are in a constant state of flux. More work, especially on taxonomy, will be necessary to determine whether these three genera have the variety of sterols they now appear to have.

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ARTICLE

Inhibition of Cholesterol Synthesis by Cyclopropylamine Derivatives of Squalene in Human Hepatoblastoma Cells in Culture

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Two squalene derivatives, trisnorsqualene cyclopropylamine and trisnorsqualene *N*-methylcyclopropylamine, were synthesized and tested for inhibition of lanosterol and squalene epoxide formation from squalene in rat hepatic microsomes, and for the inhibition of cholesterol synthesis in human cultured hepatoblastoma (HepG2) cells. Trisnorsqualene cyclopropylamine inhibited [^{3}H]-squalene conversion to [^{3}H]squalene epoxide in microsomes (IC_{50} = 5.0 μM), indicating that this derivative inhibited squalene mono-oxygenase. Trisnorsqualene *N*-methylcyclopropylamine inhibited [^{3}H]squalene conversion to [^{3}H]lanosterol (IC_{50} = 12.0 μM) and caused [^{3}H]-squalene epoxide to accumulate in microsomes, indicating that this derivative inhibited 2,3-oxidosqualene cyclase. Cholesterol biosynthesis from [^{14}C]acetate in HepG2 cells was inhibited by both derivatives (IC_{50} = 1.0 μM for trisnorsqualene cyclopropylamine; IC_{50} = 0.5 μM for trisnorsqualene *N*-methylcyclopropylamine). Cells incubated with trisnorsqualene cyclopropylamine accumulated [^{14}C]squalene, while cells incubated with trisnorsqualene *N*-methylcyclopropylamine accumulated [^{14}C]squalene epoxide and [^{14}C]squalene diepoxide. The concentration range of inhibitor which caused these intermediates to accumulate coincided with that which inhibited cholesterol synthesis. The results indicate that cyclopropylamine derivatives of squalene are effective inhibitors of cholesterol synthesis, and that substitutions at the nitrogen affect enzyme selectivity and thus the mechanism of action of the compounds.
Lipids 27, 157–160 (1992).

Hypercholesterolemia is a major risk factor for the development of atherosclerotic vascular disease. Reducing the rate of cholesterol synthesis has been shown to be an effective therapeutic approach to lowering elevated serum cholesterol levels (1). However, the only drug currently available for the treatment of hypercholesterolemia which acts by inhibiting cholesterol synthesis is mevinolin (Lovastatin). This drug inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the pathway of cholesterol biosynthesis (2). Two other enzymes in this pathway, squalene mono-oxygenase and 2,3-oxidosqualene cyclase, are also potential therapeutic targets of drugs intended to reduce sterol synthesis (3–8). Because of their position in the biosynthetic sequence, inhibition of either of these enzymes avoids potential problems caused either by reduced synthesis of polyisoprene units or the accumulation of sterol intermediates. Also, partial inhibition of 2,3-oxidosqualene cyclase may cause cells to accumulate oxysterols (9), which act to inhibit cholesterol synthesis indirectly by reducing transcription of the gene encoding for HMG-CoA reductase and by increasing the rate of HMG-CoA reductase degradation (10). We report here that two structurally similar squalene analogues inhibit these enzymes. Trisnorsqualene cyclopropylamine inhibits microsomal squalene mono-oxygenase, and trisnorsqualene *N*-methylcyclopropylamine inhibits microsomal 2,3-oxidosqualene cyclase. Furthermore, we show that both compounds inhibit cholesterol biosynthesis in a human cultured hepatoblastoma cell line, and that this inhibition apparently occurs because of blockade of the respective targeted enzyme.

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Abbreviations: FBS, fetal bovine serum; HBSS, Hanks buffered salt solution; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HPLC, high-performance liquid chromatography; LPDS, lipoprotein-deficient bovine serum; MEM, minimal essential medium.

MATERIALS AND METHODS

Materials. Male Sprague Dawley rats were purchased from Harlan (Indianapolis, IN). Human hepatoblastoma cells (HepG2; ATCC # HB8065) were purchased from American Type Culture Collection (Rockville, MD). Fetal Bovine Serum (FBS), Eagle's Minimal Essential Medium (MEM), T-160 culture flasks and 24-well cluster plates, Hanks Balanced Salt Solution (HBSS), MEM non-essential amino acids (10 mM), trypsin/EDTA (0.25%/1.0 mM), penicillin, streptomycin and glutamine (200 mM) were purchased from GIBCO (Grand Island, NY). Lipoprotein-deficient bovine calf serum (LPDS) was from Biomedical Technologies Inc. (Stoughton, MA). Cholesterol, squalene, lanosterol, FAD, NADPH and Tween-80 were from Sigma (St. Louis, MO). Methanol, hexane, isopropanol and tetrahydrofuran were purchased from Burdick and Jackson (Muskegan, MI). Flo-Scint II scintillation fluid was from Radiomatic (Tampa, FL). [7-^{3}H(N)]Cholesterol (21.0 Ci/mmole), [4,8,12,13,17,21-^{3}H]squalene (28.9 Ci/mmole) and [2-^{14}C]acetic acid (57 mCi/mmole) were from DuPont/New England Nuclear (Wilmington, DE). [^{3}H]Squalene was purified by high-performance liquid chromatography (HPLC) prior to use. Trisnorsqualene cyclopropylamine was prepared by reductive amination of trisnorsqualene aldehyde with cyclopropylamine (8). Subsequent reduction amination of formaldehyde with trisnorsqualene cyclopropylamine gave the expected *N*-methyl derivative. The products were purified by flash chromatography on silica gel. Structures obtained were consistent with spectral data.

Enzyme assays. Rat hepatic microsomes were used as the source of enzymes (11). Squalene mono-oxygenase activity was determined by monitoring the conversion of [^{3}H]squalene to [^{3}H]squalene epoxide, and 2,3-oxidosqualene cyclase activity was determined by monitoring the conversion of [^{3}H]squalene to [^{3}H]lanosterol (11). Trisnorsqualene cyclopropylamine and trisnorsqualene *N*-methylcyclopropylamine were dissolved in methanol and mixed with 100 μL of a solution of 0.1% Tween-80 and 0.6 mM [^{3}H]squalene (sp. act. 5,000 dpm/nmole) in

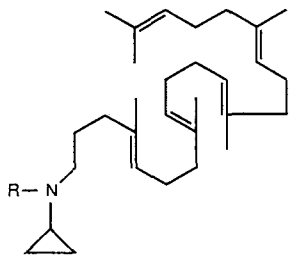
acetone. The solvents were evaporated under N_2 gas, and the residue was dissolved in a mixture of 1.0 mg microsomal protein, 7.0 mg cytosolic protein and 0.01 mM FAD in 0.1 M Tris-HCl, pH 7.4. The volume was adjusted to 950 μL with Tris buffer, and tubes were pre-incubated for 10 min at 37°C. Fifty μL of buffer were added to tubes designated as blanks, and 50 μL of 20 mM NADPH in buffer was added to all other tubes. All tubes were then incubated at 37°C for 45 min. Following incubation, 1.0 mL water was added and products were extracted twice, each time into 2.0 mL hexane. The hexane extracts were combined and dried under N_2 gas. The residue was dissolved in 60 μL tetrahydrofuran/methanol (1:1, v/v) and finally transferred to 200 μL minivials for HPLC analysis.

High-performance liquid chromatography. Samples (45 μL) were injected onto a 3.9 × 300 mm Waters μBondapak ODS column (Waters Associates, Milford, MA) and fractionated isocratically using a solvent of 7% water in methanol delivered at 1.5 mL/min. Peaks were monitored at 200 to 350 nm with a Waters 990 diode array detector, and for radioactivity with a Radiomatic FLO-1β flow-through scintillation detector (Radiomatic) connected in series with the diode array detector. Counting efficiencies were determined by injecting known amounts of either [^{3}H]- or [^{14}C]cholesterol into the HPLC system without an attached column but otherwise using conditions identical to those used for samples. Counting efficiencies were 23% for ^{3}H and 49% for ^{14}C. Recovery of [^{3}H]cholesterol from cell extracts (see below) was 91%.

Cell culture. Stock cultures of HepG2 cells were treated for 5.0 min with trypsin/EDTA at room temperature to detach the cells. The cells were suspended in MEM containing 1.0 mM non-essential amino acids, 2.0 mM L-glutamine, 1.0 mM pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin and 10% FBS. They were seeded at 3 × 10^5 cells/well into 24-well cluster dishes and incubated with 1.0 mL medium at 37°C in an atmosphere of 5% CO_2 + 95% air for 24 hr. The cells were then washed twice with HBSS, the medium was replaced with one that contained 5% LPDS instead of FBS, and the cells were incubated for 17 hr prior to the start of an experiment. Drugs were added in 10 μL ethanol to the media. Control cultures received 10 μL of ethanol. Four hours later, 4 × 10^5 dpm of [^{14}C]acetate was added, and the cells were allowed to incubate for an additional 18 hr. The cells were then washed, and 1.0 mL of hexane/isopropanol (3:2, v/v) was added to each well to extract cell lipids. Squalene, squalene monoepoxide, squalene diepoxide (15 μg each), cholesterol and lanosterol (60 μg each) were added to the extracts. [^{3}H]Cholesterol (5,000 dpm) was also added to extracts as a radioactive standard to estimate recovery and to correct for differences in retention times between the ultraviolet (UV) and radioisotope detectors caused by their physical separation. The extracts were dried under N_2 gas, and redissolved in 1.0 mL of 5% KOH in 90% ethanol. The extracts were then incubated for 60 min at 70°C. One mL water was added, and the nonsaponified material was extracted twice, each time into 2.0 mL hexane. The hexane extracts were dried, and the residue was finally dissolved in THF/M for HPLC analysis. Identification of metabolites was based on their retention times against authentic standards. The cell protein residue left in the culture wells after lipid extraction was dissolved in 0.3 N NaOH at 4°C overnight. The protein content of this solution was measured by the method of Lowry *et al.* (12). Results (dpm/peak) were normalized to cell protein, and expressed as relative proportions of total radioactive metabolites. Cell toxicity was estimated by light microscopic examination of the cultures and by measurements of cell protein per well. Drug-treated cultures were indistinguishable from controls.

RESULTS

Trisnorsqualene *N*-methylcyclopropylamine (Fig. 1) increased [^{3}H]squalene epoxide and decreased [^{3}H]-



R= H; trisnorsqualene cyclopropylamine

R= CH_3; trisnorsqualene N-methylcyclopropylamine

FIG. 1. Structures of trisnorsqualene cyclopropylamine and trisnorsqualene *N*-methylcyclopropylamine.

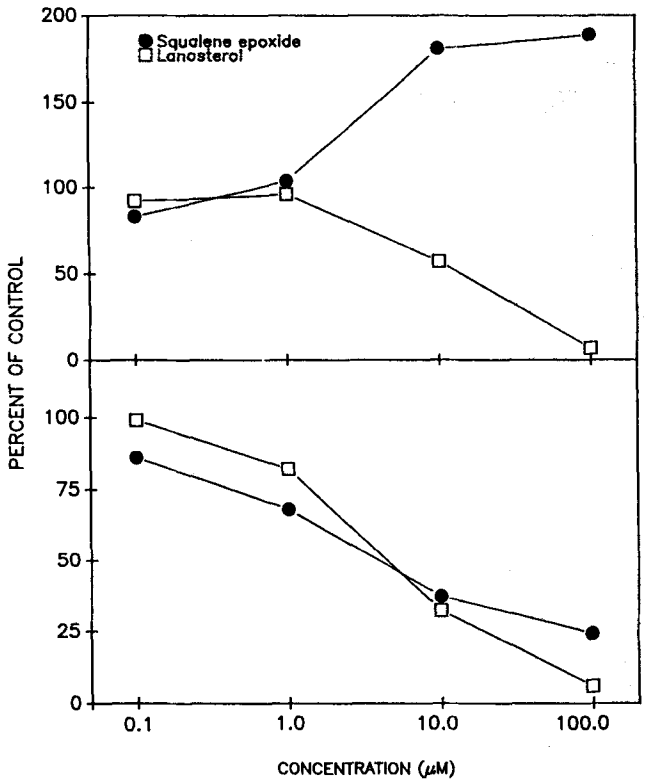


FIG. 2. Effects of increasing concentrations of either trisnorsqualene *N*-methylcyclopropylamine (top panel) or trisnorsqualene cyclopropylamine (bottom panel) on [^{3}H]squalene conversion to [^{3}H]squalene monoepoxide and [^{3}H]lanosterol by rat hepatic microsomes.

lanosterol production in the microsomal assay, both effects indicating that this compound inhibited 2,3-oxidosqualene cyclase (Fig. 2, top panel). The effect of the compound was dose-dependent, as shown in Figure 2, top panel. The calculated 50% of inhibitory concentration (IC_{50}) for inhibition of lanosterol synthesis was 12 μM. Trisnorsqualene cyclopropylamine (Fig. 1) inhibited [^{3}H]squalene conversion to [^{3}H]squalene epoxide (Fig. 2, bottom panel), indicating that it inhibited squalene mono-oxygenase. This compound also inhibited lanosterol synthesis (Fig. 2, bottom panel) as expected, since it blocked formation of the substrate for 2,3-oxidosqualene cyclase. Trisnorsqualene cyclopropylamine also acted in a dose-dependent manner (Fig. 2, bottom panel). The calculated IC_{50} for inhibition of squalene epoxide synthesis was 5 μM.

Because trisnorsqualene cyclopropylamine and its *N*-methyl analogue inhibited squalene mono-oxygenase and 2,3-oxidosqualene cyclase, respectively, in hepatic microsomes, it was of interest to determine if these compounds would affect the same enzymes and block cholesterol biosynthesis in intact hepatocytes. Trisnorsqualene cyclopropylamine caused an accumulation of squalene in HepG2 cells at concentrations greater than 0.1 μM (Fig. 3). Associated with the squalene accumulation was a corresponding decrease in [^{14}C]cholesterol synthesis (IC_{50} = 1.0 μM). At concentrations greater than 1.0 μM squalene monoepoxide accumulated, indicating that trisnorsqualene cyclopropylamine may also inhibit 2,3-oxidosqualene cyclase. Neither squalene diepoxide nor lanosterol levels exceeded background levels over the concentration range tested.

Trisnorsqualene *N*-methylcyclopropylamine also caused a dose-dependent inhibition of [^{14}C]cholesterol synthesis as shown in Figure 4 (IC_{50} = 0.5 μM). This inhibition of cholesterol synthesis was associated not with an accumulation of squalene, however, but with an accumulation of squalene mono- and diepoxide. Squalene monoepoxide levels began to increase at 0.01 μM drug concentration, and continued to rise until 1.0 μM. Squalene diepoxide levels exceeded background levels at drug concentrations greater than 0.01 μM, and at 1.0 μM and above exceeded those of squalene monoepoxide. These results

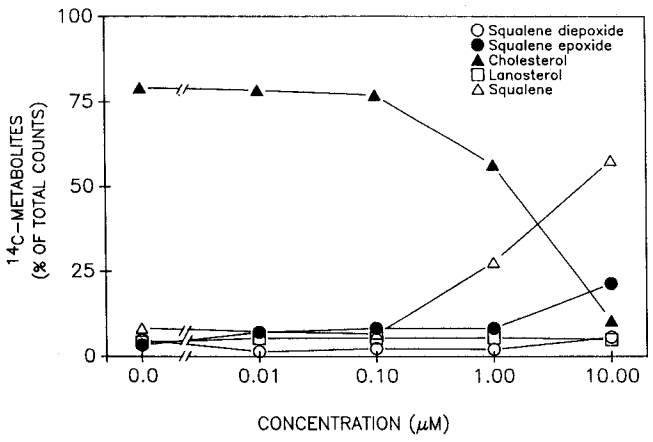


FIG. 3. Effects of increasing concentrations of trisnorsqualene cyclopropylamine on the conversion of [^{14}C]acetate to the indicated ^{14}C-labeled metabolites in cultured HepG2 cells.

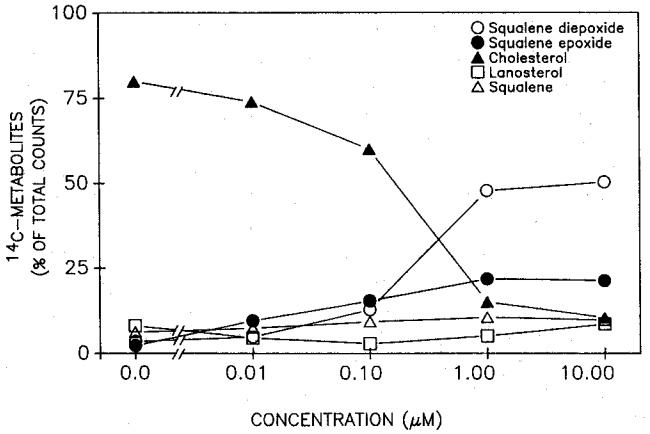


FIG. 4. Effects of increasing concentrations of trisnorsqualene *N*-methylcyclopropylamine on the conversion of [^{14}C]acetate to the indicated ^{14}C-labeled metabolites in cultured HepG2 cells.

suggest that this compound inhibited 2,3-oxidosqualene cyclase rather than squalene mono-oxygenase.

DISCUSSION

Our results confirm a recent report (8) that trisnorsqualene cyclopropylamine inhibits microsomal squalene mono-oxygenase. We found that this compound inhibited rat hepatic microsomal squalene monooxygenase with an IC_{50} of 5 μM, a result which is similar to that for the porcine microsomal enzyme (8). In addition, we found that the *N*-methyl derivative of trisnorsqualene cyclopropylamine inhibited rat hepatic microsomal 2,3-oxidosqualene cyclase. This compound did not inhibit the porcine hepatic microsomal enzyme (8). There are no apparent reasons other than species differences which could account for this difference in inhibitory activity. However, our results showing the accumulation of squalene epoxides in cultured HepG2 cells incubated with the *N*-methyl derivative confirm the results of our microsomal enzyme assay.

The two compounds studied here differ only in a methyl substitution on the nitrogen, yet they inhibited two different enzymes, both in hepatic microsomes and cultured hepatocytes. Trisnorsqualene cyclopropylamine and its *N*-methyl derivative are secondary and tertiary amines, respectively, and this structural difference appears to account for the difference in enzyme specificity. Trisnorsqualene cyclopropylamine could inactivate squalene mono-oxygenase through a mechanism involving aminium radical formation, followed by cyclopropane ring opening and alkylation of the enzyme's active site. Such a mechanism has been proposed to account for inhibition of monoamine oxidase and cytochrome P-450 by cyclopropylamines (13). Alkylation by trisnorsqualene cyclopropylamine is unlikely, however, because it has been shown that inhibition of porcine squalene mono-oxygenase by this compound is reversible (8). The *N*-methyl derivative, by virtue of being a positively charged tertiary amine at pH 7.4, may mimic a carbocationic intermediate formed during formation of the A ring of lanosterol and thereby competitively inhibit 2,3-oxidosqualene cyclase (14–16). The *N*-methyl derivative neither inhibited microsomal squalene mono-oxygenase, nor did it cause squalene accumulation in HepG2 cells. Considering the inhibition of

squalene mono-oxygenase by trisnorsqualene cyclopropylamine, it is not clear why the *N*-methylcyclopropylamine derivative did not also inhibit squalene mono-oxygenase, though the methyl group may sterically hinder access of the compound to the enzyme's active site.

When HepG2 cells were incubated with trisnorsqualene *N*-methylcyclopropylamine, squalene diepoxide accumulated to levels exceeding those of squalene monoepoxide (see Fig. 4). This is consistent with the ability of squalene mono-oxygenase, or another polyisoprene oxygenase, to catalyze an epoxidation of squalene monoepoxide to form the diepoxide in intact cells. Therefore, in the presence of an inhibitor of 2,3-oxidosqualene cyclase, such as trisnorsqualene *N*-methylcyclopropylamine, squalene diepoxide rather than squalene monoepoxide is the terminal product.

Trisnorsqualene cyclopropylamine caused squalene monoepoxide to accumulate in the HepG2 cells, although at concentrations 10-fold higher than those required to cause squalene accumulation. This suggests that the compound inhibits 2,3-oxidosqualene cyclase, but with a potency at least 10-fold less than for inhibition of squalene mono-oxygenase. It also indicates that squalene mono-oxygenase is incompletely inhibited at these concentrations. Inhibition of 2,3-oxidosqualene cyclase by trisnorsqualene cyclopropylamine would be consistent with the known ability of various azasqualene analogues to inhibit this enzyme (3).

Both compounds inhibited cholesterol synthesis in HepG2 cells. Associated with this inhibition was cellular accumulation of the substrates for squalene mono-oxygenase and 2,3-oxidosqualene cyclase when the cells were incubated with trisnorsqualene cyclopropylamine and trisnorsqualene *N*-methylcyclopropylamine, respectively. [^{14}C]Acetate would not have incorporated into these substrates if the inhibition of cholesterol synthesis was due to blockade of an enzyme(s) upstream in the reaction sequence to either of the two target enzymes. Furthermore, for each compound the curve describing the decrease in cholesterol synthesis overlapped the curve describing enzyme substrate accumulation. If inhibition of cholesterol synthesis was due to blockade of enzyme(s) downstream in the reaction sequence to the target enzymes, these curves would have been separated due to the higher drug concentration required to block the target enzymes relative to the downstream step(s). This correlation of substrate accumulation with inhibition of cholesterol synthesis, however, does not prove that other enzymes in the cholesterol biosynthetic sequence are unaffected. Nevertheless, these results combined with those showing direct inhibition of microsomal enzymes suggest that trisnorsqualene cyclopropylamine and trisnorsqualene *N*-methylcyclopropylamine inhibit cholesterol synthesis in cells by mechanisms involving the inhibition of squalene mono-oxygenase and 2,3-oxidosqualene cyclase, respectively.

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Effects of n-3 and n-6 Fatty Acids on Tumor Necrosis Factor Cytotoxicity in WEHI Fibrosarcoma Cells

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Modulation by fatty acids of the cytotoxic effect of recombinant tumor necrosis factor alpha (TNF) toward WEHI 164 mouse fibrosarcoma cells has been examined. Preincubating the highly TNF-sensitive WEHI clone 13 cells for 44 hr with 50 μmol/L of 20:5n-3, 22:6n-3, 18:3n-6, 20:3n-6 or 20:4n-6 reduced cell survival 22 hr after challenge with TNF (40 ng/L) by 65%, 72%, 60%, 98% and 85%, respectively. In comparison, 18:3n-3, 18:2n-6 and 18:1n-9 had only negligible effects on TNF-induced toxicity. Different extent of fatty acid incorporation into cell total phospholipids or triglycerides could not explain the observed effects on TNF cytotoxicity, and the enhanced cytotoxicity could therefore not be explained merely by an increased unsaturation of the cell membranes. In addition to the fatty acid supplied, preincubation with 18:2n-6, 18:3n-6 or 18:3n-3 also enriched the cells with 20:2n-6, 20:3n-6 and 20:3n-3, respectively, most likely due to chain elongation. The results suggest that the WEHI cells have a low Δ6 desaturase activity, and that n-6 and n-3 acids must have at least 3 or 4 double bonds, respectively, to enhance TNF cytotoxicity in WEHI cells. Dexamethasone partly inhibited TNF-induced cytotoxicity, while cyclooxygenase, thromboxane synthetase or lipoxygenase inhibitors had no or negligible effects. The antioxidant butylated hydroxyanisole (BHA) completely inhibited TNF-induced cytotoxicity, while the structurally and functionally similar antioxidant butylated hydroxytoluene had no such effect, indicating that BHA does not block TNF cytotoxicity through its antioxidant effect. The results suggest that TNF cytotoxicity involves, directly or indirectly, metabolism of long-chain polyunsaturated fatty acids, and we speculate that fatty acid metabolites are involved.
Lipids 27, 161-168 (1992).

Human mononuclear phagocytes mediate a large number of immunological responses, and are probably part of the immunological defense against cancer *in vivo* (1). Tumor necrosis factor (TNF) seems to be a major effector molecule in the monocyte-mediated lysis of cancer cells *in vitro* (2), and recombinant TNF depresses growth and kills some sensitive cancer cells *in vitro* (3). Studies by Urban *et al.* (4) indicate that cancer cells resistant to TNF are resistant to macrophage-mediated killing. Both TNF-sensitive and -resistant cells have high-affinity receptors for TNF (5). Activators of protein kinase C decrease the number of TNF receptors on the cell surface (6), thereby inhibiting TNF-mediated cytotoxicity. The observed difference between cancer cell lines in sensitivity to TNF has partly been attributed to high-molecular-weight components in the TNF receptor itself (7), and partly to differences in the intracellular concentration of glutathione (8). The cellular mechanisms involved in TNF-mediated cytotoxicity are largely unknown, although several reports indicate that 20:4n-6 metabolism is involved (9,10). It has been shown that TNF induces release of 20:4n-6 from cell membrane phospholipids, and also that some phospholipase A_2 inhibitors protect against TNF-induced cytotoxicity (11). This indicates that activation of phospholipase(s) may be involved in TNF-induced cytotoxicity.

Studies from Japan and Greenland eskimos suggest that a diet enriched in n-3 fatty acids is related to a low incidence of breast and colon cancer (12). In addition, animal studies have indicated that the growth of some tumors is affected by the fatty acid composition of the diet (13,14). It has also been found that the fatty acid composition of cancer cells affects their sensitivity to antibody plus complement mediated killing *in vitro* (15). These observations could indicate that the fatty acid composition of cancer cells' membrane lipids might affect their sensitivity to TNF-mediated cytotoxicity. We have therefore enriched the TNF-sensitive WEHI 164 fibrosarcoma cell line with saturated and unsaturated fatty acids, and investigated how this affects the cellular sensitivity to TNF.

MATERIALS AND METHODS

Materials. RPMI-1640, fetal calf serum, L-glutamine, trypsin solution, gentamicin sulfate, actinomycin D, sodium dodecyl sulfate (SDS), and Dulbecco's phosphate buffered saline were obtained from Gibco (Paisley, U.K.). Oleic, alpha-linolenic, eicosapentaenoic, arachidonic, linoleic, gamma-linolenic and palmitic acid, dexamethasone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nordihydroguaretic acid (NDGA), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were obtained from Sigma (St. Louis, MO). Docosahexaenoic and dihomogamma-linolenic acids were purchased from Nu Chek Prep. (Elysian, MN). The purity of all fatty acids was better than 95% (w/w). Dichloro-(R)fluorescein was from B.D.H. Lab., Chemicals Division (Poole, England). Adsorbosil plus-1 softlayer preadsorbent thin-layer chromatography (TLC) plates and the SGE BPX 70 capillary column were obtained from Alltech (Deerfield, IL). Absolute ethanol was obtained from Vinmonopolet A/S (Oslo, Norway). Ascorbic acid, methanolic base and the SP2330 capillary column were from Supelco (Bellefonte, PA). Chloroform, n-hexane, isooctane, toluene, isopropanol and methanol of chromatographic quality were obtained from E. Merck (Darmstadt, Germany). Diethyl ether and acetic acid of p.a. quality were also obtained from E. Merck. Recombinant murine TNF alpha

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Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; ED_{50}, defined as the TNF concentration that reduces cell density by 50%; FAME, fatty acid methyl ester(s); FCS-M, RPMI-1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 2 mmol/L L-glutamine and 40 mg/L gentamicin; GLC, gas-liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDGA, nordihydroguaretic acid; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; TNF, recombinant murine tumor necrosis factor alpha.

(8×10^7 U/mg protein) was generously provided by Genentech Inc. (San Francisco, CA).

Cell culture. The WEHI 164 cell line (termed WEHI parental cells) was obtained from Dr. H. W. Löms Ziegler-Heitbrock (University of Munich, Germany). The highly TNF-sensitive WEHI 164 clone 13 cells (WEHI clone 13) were previously isolated (16) from the WEHI parental cells. WEHI 164 parental and clone 13 cells were grown in RPMI-1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 2 mmol/L L-glutamine and 40 mg/L gentamicin (FCS-M). The cells were grown in 95% air and 5% CO_2 in a humidified atmosphere. Fatty acids dissolved in ethanol were added as indicated; the final ethanol concentration was 0.025% (v/v). Controls containing 0.025% ethanol were always included and had no effect on TNF cytotoxicity. Concentration and purity of the ethanolic fatty acid stock solutions were checked by capillary gas-liquid chromatography (GLC) after methylation with BF_3/methanol (17) using nonadecanoic acid as internal standard.

Viability. After incubation of the WEHI cells with 50 μmol/L of different fatty acids in FCS-M for 44 hr, the viability was at least 95% as determined by trypan blue exclusion.

MTT assay. A modification of the method described by Mosmann (18) was used. If not otherwise indicated, cells were cultured in 75-cm^2 flasks (Costar 3275), trypsinized and seeded at a density of 2×10^3 cells/well in 96-well microplates (Costar 3598) using 100 μL medium/well. After 4-hr incubation, 100 μL FCS-M containing the appropriate fatty acid supplement was added. Half the medium was replenished after 22 hr to avoid depletion of the added fatty acid, and cells were tested for TNF sensitivity after a total preincubation time of 44 hr. At this time, all cells were in logarithmic phase of growth (results not shown). Cell growth was slightly suppressed by 50 μmol/L of 22:6n-3, 20:5n-3, 20:4n-6 and 20:3n-6 both when measured with the MTT assay and by cell counting using a Coulter ZF cell counter (Coulter Electronics LTD, Dunstable Beds, England). After 44-hr preincubation, the medium was changed to 200 μL fatty acid-free FCS-M containing TNF as indicated. Cell survival was then measured after 22-hr further incubation, using the MTT assay as previously described (16). Wells containing medium but no cells were used to correct for background absorbance. Cell survival was calculated as: 100 × (OD in wells with TNF)/(OD in wells without TNF).

To test whether fatty acid enrichment of WEHI cells affected the MTT assay, cells were preincubated with 50 μmol/L fatty acid or 0.025% (v/v) ethanol for 44 hr. The cells were then transferred into fatty acid-free FCS-M and 22 hr later seeded into microplates containing FCS-M and 1 mg/L of actinomycin D. The MTT assay was then performed immediately. Neither ethanol, 18:1n-9, 20:4n-6, 20:5n-3, nor 22:6n-3 without TNF had any effect on the MTT assay (results not shown).

Chromium release assay. The chromium release assay was a modification of the method described by Brunner *et al.* (19). WEHI clone 13 cells were trypsinized, and 1×10^6 cells were seeded as non-adherent cultures in 1400 mm microbiological petri dishes (Nunc A/S, Roskilde, Denmark) in 10 mL FCS-M. Four hours later, the indicated fatty acid was added to the cells in a total volume of 20 mL FCS-M. After preincubation with fatty acids for 44 hr, cells were pelleted at +4°C (200 × *g*, 8 min), and resuspended in FCS-M (+4°C). Thereafter, 4×10^6 cells were incubated with 150 μCi $Na_2{}^{51}CrO_4$ (specific activity of 11 GBq/mg chromium, Institutt for Energiteknikk, Kjeller, Norway) for 60 min at 37°C in a total volume of 0.75 mL. The cells were then washed three times in +4°C FCS-M (200 × *g*, 8 min). The labeled cells were seeded at 1×10^4 cells/well in microplates (Costar 3598) in 200 μL of medium containing TNF and/or the test compounds. The supernatants were harvested after 16-hr further incubation using a supernatant collecting system (Skatron A/S, Lier, Norway). Percent specific lysis was calculated as: $100 \times (E - S)/(T - S)$, where E is cpm released from cells added TNF, S is cpm released spontaneously from cells added medium only, and T is cpm released after complete lysis of the cells added 50 μL 2.5% (w/v) of SDS.

Fatty acid analysis. Total lipids from fetal calf serum were extracted with n-butanol and separated into phospholipids, free fatty acids and neutral lipids on Bond Elut aminopropyl columns (Varian SPP., Harbor City, CA) after adding diheptadecanoylglycerophosphocholine, nonadecanoic acid and trinonadecanoin as internal standards and BHT as antioxidant (20). Phospholipids were transmethylated with BF_3/methanol in Teflon-lined screw-capped tubes filled with N_2, at 100°C for 30 min, and fatty acid methyl esters (FAME) were extracted into isooctane and analyzed by GLC as previously described (21). Free fatty acids were methylated with BF_3/methanol, extracted into hexane, and dissolved in isooctane after evaporation under N_2. Neutral lipids were taken to dryness by N_2, dissolved in toluene, and transmethylated with methanolic base at 80°C for 20 min. The reaction was stopped by addition of water. After extraction into n-hexane and evaporation under N_2, FAME were dissolved in isooctane and analyzed by GLC.

Triglyceride and total phospholipid fatty acids in WEHI clone 13 cells were quantitated after washing the cells twice at +4°C in Dulbecco's phosphate buffered saline (without calcium and magnesium) and extracting the lipids according to Bligh and Dyer (22). BHT (10 μg) was added to each sample as antioxidant, and trinonadecanoin and diheptadecanoylglycerophosphocholine were used as internal standards. Lipid classes were separated on TLC plates activated at 110°C for one hour and prewashed with hexane/diethyl ether (80:20, v/v). Triglycerides and total phospholipids were separated using hexane/diethyl ether/acetic acid (80:20:1, v/v/v), containing 0.001% (w/v) BHT, as solvent (23). The lipids were made visible under ultraviolet (UV)-light after spraying with 0.001% (w/v) dichlorofluorescein in ethanol. The phospholipid and triglyceride fractions were identified by comparison with standards, scraped into Teflon-capped tubes, and transmethylated with methanolic base for 20 min at 80°C under N_2; FAME were thereafter extracted twice into n-hexane, taken to dryness under a stream of N_2, and finally dissolved in isooctane. One μL of the mixture was injected into the Hewlett-Packard 5890A GLC (Avondale, PA) equipped with a SP2330 capillary column as previously described (21), but using a splitless injection technique. The SP2330 column does not separate 20:4n-6 and 20:3n-3. The samples were therefore reanalyzed on a SGE BPX 70 column to calculate the content of 20:4n-6 and 20:3n-3. Initial temperature was 80°C on both columns. The purge vent was opened after 3 min, and after 3.2 min the

temperature was programmed at 40°C/min to 170°C. After a 1 min hold, the temperature was programmed at 1.5°C/min to 210°C. Total run time was 52 min.

Statistics. Analysis of variance (ANOVA) was performed using the SPSS-PC+ version 4.0 (SPSS Inc., Chicago, IL) or the SAS statistical packages version 6.0 (SAS Institute Inc., Cary, NC).

RESULTS

Preincubation of WEHI 164 clone 13 cells with 12.5 to 50 μmol/L of different fatty acids influenced the cellular sensitivity to TNF-induced cytotoxicity. When 40 ng/L of TNF was added to cells preincubated with fatty acid free FCS-M, cell survival decreased to 43% (Fig. 1). In comparison, preincubation with 50 μmol/L 20:3n-6 or 20:4n-6 followed by addition of TNF, decreased cell survival to 1 and 7%, respectively. Their precursor 18:3n-6 was equally effective at 25 μmol/L, but slightly less effective than 20:3n-6 and 20:4n-6 at 50 μmol/L. In comparison, 20:5n-3 and 22:6n-3 were slightly less effective than 20:3n-6 and 20:4n-6, decreasing cell survival to 15 and 12% at 50 μmol/L, respectively. Preincubating the cells with 18:1n-9, 18:3n-3 or 18:2n-6 had negligible effects on TNF-induced toxicity, while both 16:0 and 20:1n-9 at 50 μmol/L decreased cell survival to approximately 34%.

Preincubation of WEHI clone 13 cells with 20:4n-6 increased the sensitivity to TNF-induced cytotoxicity in a time-dependent way. The WEHI clone 13 cells received either control medium, 18:2n-6 or 20:4n-6 at increasing time intervals before challenge with TNF (Fig. 2). Preincubation with 50 μmol/L 20:4n-6 decreased cell survival from 64% to 45%, 34% and 26% after 24, 48 and 72 hr preincubation time, respectively. In comparison, preincubation with control medium or 18:2n-6 had no or only small effects. In this experiment, 18:2n-6 or 20:4n-6 was also added simultaneously with TNF to cells that had been preincubated with control medium only. Again, 18:2n-6 had no effect, while 20:4n-6 reduced cell survival from 64% to 55% (Fig. 2). The WEHI cells preincubated in control medium only were more resistant to TNF at day 3 than day 2 after seeding, explaining the higher survival of control cells in this experiment compared to Figure 1.

The fatty acid composition of total phospholipids and triglycerides in WEHI clone 13 cells preincubated with 50 μmol/L of different fatty acids is shown in Tables 1 and 2, respectively. Fatty acid analysis showed that enrichment with 18:1n-9 and 18:2n-6 slightly decreased the concentration of 20:3n-6 and 20:4n-6 in cell phospholipids. Enrichment with 18:3n-6 increased 20:3n-6 in both phospholipids and triglycerides nearly as much as enrichment with 20:3n-6, while 18:3n-6 increased 20:4n-6 significantly only in the triglyceride fraction. In comparison, 18:3n-3 increased 20:3n-3 and decreased both 20:3n-6 and 20:4n-6 in total phospholipids. Interestingly, 22:6n-3 had no effect on the level of 20:3n-6 or 20:4n-6, while 20:5n-3 decreased both 20:3n-6 and 20:4n-6 in total phospholipids. Preincubation with 20:4n-6 decreased both 20:5n-3 and 22:6n-3 in the total phospholipids. With a few exceptions, the triglyceride fatty acids changed rather similarly as did the phospholipid fatty acids (Table 2). While 18:3n-3 increased 18:3n-3, 20:3n-3 and 20:5n-3, but decreased 22:6n-3 in the phospholipid fraction, it increased both 18:3n-3, 20:3n-3, 20:5n-3 and 22:6n-3 in the triglycerides. Further, preincubation with 20:5n-3 increased 20:5n-3 and 22:5n-3 to the same extent, and at the same time reduced 22:6n-3 in the phospholipid fraction, while both 22:5n-3 and 22:6n-3 increased in the triglycerides.

Fatty acid analysis showed that the unsupplemented FCS-M contained very low concentrations of both n-3 and n-6 fatty acids in the free fatty acid fraction, where 16:0, 18:0 and 18:1n-9 comprised 74% of total free fatty acids (Table 3). In comparison, the total n-3 and total n-6 fatty acids comprised 7.5% and 11.2%, respectively. A similar

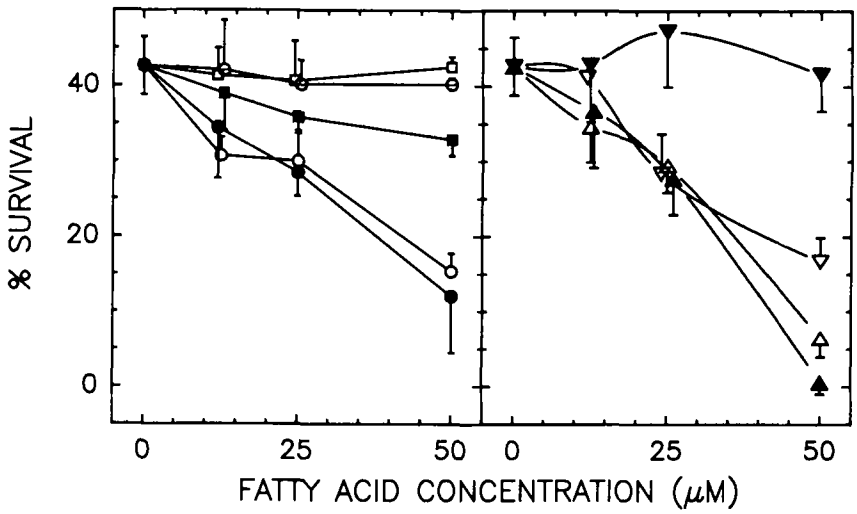

FIG. 1. Effect of different fatty acids on TNF-induced cytotoxicity in WEHI 164 clone 13 cells. Cells were preincubated for 44 hr with or without fatty acids at the concentrations indicated. TNF (40 ng/L) was then added, and percent cell survival was quantitated by the MTT assay as described in Materials and Methods. Results are from one of two similar experiments and are given as the mean of quadruplicate determinations, with SD shown as vertical bars. A: Effect of 16:0 (■), 18:1n-9 (□), 18:3n-3 (⊖), 20:5n-3 (○) and 22:6n-3 (●). B: Effect of 18:2n-6 (▼), 18:3n-6 (▽), 20:3n-6 (▲) and 20:4n-6 (△).

TABLE 1

Fatty Acid Composition of Total Phospholipids in WEHI Clone 13 Cells: Effect of Fatty Acid Supplementation

Fatty acid measured (% of total)	Fatty acid added[a]								
	None	18:1n-9	18:2n-6	18:3n-6	20:3n-6	20:4n-6	18:3n-3	20:5n-3	22:6n-3
16:0	20.9 ± 1.76	11.1 ± 0.40	13.3 ± 6.81	24.6 ± 0.13	21.9 ± 2.94	31.4 ± 0.72	18.5 ± 0.39	35.0 ± 2.32	31.8 ± 2.68
18:0	14.8 ± 0.27	9.0 ± 0.18	12.9 ± 1.34	11.8 ± 0.29	11.6 ± 1.95	13.0 ± 0.16	14.6 ± 0.18	15.4 ± 0.76	17.5 ± 0.31
18:1n-9	35.4 ± 0.40	61.4 ± 1.01	10.7 ± 0.36	12.9 ± 0.09	14.0 ± 2.13	14.6 ± 0.07	13.1 ± 0.20	18.0 ± 0.64	22.1 ± 0.16
20:1n-9	2.7 ± 0.26	4.0 ± 0.10	0.9 ± 0.26	0.5 ± 0.01	0.6 ± 0.12	0.6 ± 0.02	0.8 ± 0.03	0.6 ± 0.02	0.8 ± 0.05
18:3n-3	0.12 ± 0.02	0.03 ± 0.01	0.05 ± 0.02	0.04 ± 0.005	0.07 ± 0.02	0.07 ± 0.004	28.53 ± 0.30	0.19 ± 0.08	0.27 ± 0.01
20:3n-3	0.16 ± 0.03	0.08 ± 0.01	0.13 ± 0.03	0.02 ± 0.04	0.10 ± 0.02	0.12 ± 0.08	7.84 ± 0.11	0.11 ± 0.08	0.12 ± 0.06
20:5n-3	0.52 ± 0.02	0.24 ± 0.01	0.09 ± 0.03	0.11 ± 0.005	0.06 ± 0.01	0.05 ± 0.01	0.79 ± 0.02	10.3 ± 1.75	0.98 ± 0.18
22:5n-3	3.3 ± 0.69	1.5 ± 0.03	1.8 ± 0.73	1.1 ± 0.03	0.9 ± 0.23	0.7 ± 0.01	2.3 ± 0.02	8.5 ± 1.98	1.0 ± 0.22
22:6n-3	3.4 ± 0.68	1.5 ± 0.10	1.6 ± 0.64	0.7 ± 0.06	0.6 ± 0.14	0.4 ± 0.02	1.0 ± 0.04	0.4 ± 0.08	11.1 ± 2.55
18:2n-6	3.1 ± 0.08	1.4 ± 0.09	35.1 ± 1.45	1.6 ± 0.10	1.4 ± 0.21	1.5 ± 0.02	2.1 ± 0.02	1.9 ± 0.04	2.7 ± 0.10
18:3n-6	0.05 ± 0.005	0.01 ± 0.01	0.05 ± 0.004	9.84 ± 0.18	0.25 ± 0.13	0.09 ± 0.002	0.01 ± 0.01	0.02 ± 0.02	0.03 ± 0.005
20:2n-6	0.46 ± 0.04	0.23 ± 0.01	12.40 ± 3.97	0.13 ± 0.03	0.24 ± 0.05	0.17 ± 0.01	0.21 ± 0.01	0.11 ± 0.01	0.19 ± 0.01
20:3n-6	1.1 ± 0.09	0.6 ± 0.01	1.1 ± 0.32	22.6 ± 0.11	27.5 ± 5.73	0.7 ± 0.07	0.5 ± 0.002	0.6 ± 0.02	1.1 ± 0.12
20:4n-6	6.0 ± 0.33	4.3 ± 0.06	4.6 ± 1.19	7.9 ± 0.11	6.3 ± 1.33	19.8 ± 0.40	3.9 ± 0.08	1.6 ± 0.08	4.3 ± 0.56
22:4n-6	2.2 ± 0.45	1.4 ± 0.04	3.1 ± 1.26	1.6 ± 0.05	2.3 ± 0.57	11.8 ± 0.39	1.4 ± 0.04	0.3 ± 0.06	0.5 ± 0.07
22:5n-6	0.19 ± 0.03	0.08 ± 0.01	0.09 ± 0.03	0.06 ± 0.02	0.06 ± 0.002	0.04 ± 0.004	0.18 ± 0.03	0.01 ± 0.02	0.05 ± 0.02
Total phospholipid fatty acids ($\mu g/10^6$ cells)	10.4 ± 1.8	13.1 ± 1.8	10.4 ± 1.1	13.7 ± 0.3	12.0 ± 2.7	12.4 ± 0.5	11.0 ± 2.0	12.4 ± 0.5	10.6 ± 1.9

[a]WEHI clone 13 cells were incubated for 44 hr in FCS-M supplemented with 50 μM fatty acids as indicated. Results are expressed as % of total fatty acids or as $\mu g/10^6$ cells. Minor fatty acids are not listed. Mean ± SD from one experiment performed in triplicate.

TABLE 2

Fatty Acid Composition of Total Triglycerides in WEHI Clone 13 Cells: Effect of Fatty Acid Supplementation

Fatty acid measured (% of total)	Fatty acid added[a]								
	None	18:1n-9	18:2n-6	18:3n-6	20:3n-6	20:4n-6	18:3n-3	20:5n-3	22:6n-3
16:0	35.5 ± 2.08	4.5 ± 0.28	6.8 ± 0.07	2.7 ± 1.18	6.3 ± 1.95	13.5 ± 1.43	13.6 ± 0.43	15.9 ± 4.93	19.1 ± 2.93
18:0	13.0 ± 0.48	2.8 ± 0.14	2.8 ± 0.07	1.4 ± 0.16	2.3 ± 0.53	3.8 ± 0.17	6.1 ± 0.04	4.4 ± 0.20	7.3 ± 0.26
18:1n-9	29.5 ± 1.30	72.7 ± 0.98	8.3 ± 0.08	5.0 ± 0.70	9.0 ± 1.83	13.7 ± 0.25	13.7 ± 0.26	13.8 ± 0.45	20.4 ± 0.47
20:1n-9	3.4 ± 0.58	8.9 ± 0.46	0.9 ± 0.04	0.2 ± 0.03	0.3 ± 0.04	0.4 ± 0.03	0.9 ± 0.10	0.3 ± 0.06	0.4 ± 0.14
18:3n-3	ND[b]	0.05 ± 0.01	0.05 ± 0.02	0.03 ± 0.01	0.05 ± 0.01	0.14 ± 0.06	37.2 ± 0.27	0.21 ± 0.02	0.32 ± 0.03
20:3n-3	0.13 ± 0.12	0.08 ± 0.07	0.10 ± 0.002	0.18 ± 0.05	0.09 ± 0.03	0.14 ± 0.02	8.13 ± 0.30	0.18 ± 0.02	0.21 ± 0.19
20:5n-3	ND	0.03 ± 0.01	0.03 ± 0.06	0.15 ± 0.03	0.11 ± 0.03	0.08 ± 0.01	0.43 ± 0.08	17.7 ± 2.06	0.40 ± 0.13
22:5n-3	1.8 ± 0.28	2.0 ± 0.20	2.4 ± 0.08	1.4 ± 0.16	1.1 ± 0.33	1.0 ± 0.05	3.6 ± 0.01	34.2 ± 3.90	4.1 ± 0.54
22:6n-3	0.8 ± 0.18	1.3 ± 0.05	1.2 ± 0.003	0.8 ± 0.08	0.7 ± 0.08	0.6 ± 0.05	1.1 ± 0.14	0.9 ± 0.11	36.6 ± 3.00
18:2n-6	1.8 ± 0.15	1.2 ± 0.05	56.3 ± 0.32	1.2 ± 0.24	1.3 ± 0.16	2.0 ± 0.05	2.3 ± 0.03	2.3 ± 0.06	2.8 ± 0.07
18:3n-6	ND	0.03 ± 0.01	0.14 ± 0.004	13.0 ± 2.60	0.38 ± 0.09	0.20 ± 0.005	ND	ND	ND
20:2n-6	0.1 ± 0.22	0.2 ± 0.04	11.5 ± 0.12	0.05 ± 0.006	0.1 ± 0.02	0.1 ± 0.007	0.4 ± 0.03	ND	ND
20:3n-6	1.9 ± 0.29	0.6 ± 0.05	2.3 ± 0.03	61.8 ± 4.31	62.0 ± 9.99	1.7 ± 0.58	1.0 ± 0.09	1.2 ± 0.06	1.5 ± 0.13
20:4n-6	0.7 ± 0.64	0.6 ± 0.03	1.1 ± 0.04	7.2 ± 0.31	3.7 ± 0.83	29.2 ± 1.00	1.3 ± 0.39	1.3 ± 0.08	0.9 ± 0.20
22:4n-6	1.6 ± 0.26	1.5 ± 0.08	3.6 ± 0.06	3.4 ± 0.49	3.4 ± 0.96	27.6 ± 0.97	1.9 ± 0.05	0.7 ± 0.06	1.5 ± 0.12
22:5n-6	0.3 ± 0.48	0.5 ± 0.41	0.2 ± 0.03	0.1 ± 0.04	0.2 ± 0.09	0.6 ± 0.14	0.6 ± 0.10	0.2 ± 0.03	ND
Total triglyceride fatty acids ($\mu g/10^6$ cells)	0.29 ± 0.04	7.0 ± 2.68	9.9 ± 0.73	11.4 ± 1.44	10.8 ± 3.69	8.4 ± 0.32	3.2 ± 0.45	5.2 ± 0.22	2.2 ± 0.40

[a]WEHI clone 13 cells were incubated for 44 hr in FCS-M supplemented with 50 μM fatty acids as indicated. Results are expressed as % of total fatty acids or as $\mu g/10^6$ cells, and given as mean ± SD from one experiment performed in triplicate.

[b]Not detected.

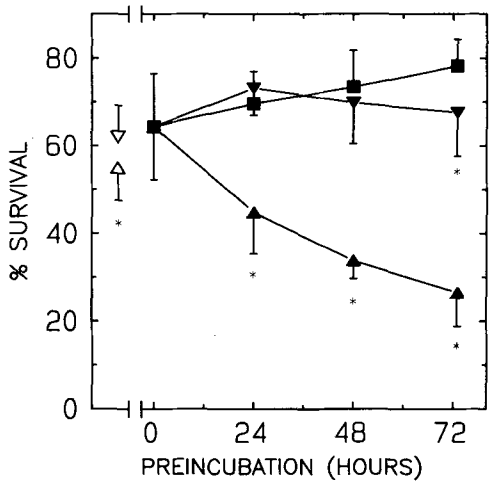

FIG. 2. Effect of preincubating WEHI clone 13 cells with 18:2n-6 and 20:4n-6. All cells were preincubated for 72 hr in microplates. At the indicated time points before challenge with TNF, the medium was changed from control medium (■,△,▽) to medium containing 50 μmol/L 18:2n-6) (▼) or 50 μmol/L 20:4n-6 (▲). At the end of preincubation, TNF (40 ng/L) was added in control medium (■,▲,▼) or in medium containing 50 μmol/L 18:2n-6 (▽) or 50 μmol/L 20:4n-6 (△). Cell survival was measured by the MTT assay 22 hr later. Results are from two experiments, each with 8 parallels, and are expressed as mean ± SD of 16 determinations. *P < 0.05 as compared to control at each time point. Data were analyzed by one-way analysis of variance using Duncan multiple comparison test.

fatty acid distribution was found in the phospholipids, while neutral lipids contained relatively more 18:1n-9 and n-6 fatty acids. The low concentrations of free fatty acids in the culture medium imply that addition of 50 μmol/L of a single fatty acid dramatically changed the composition of free fatty acids available to the cells.

Preincubation of WEHI clone 13 cells with increasing concentrations of 20:5n-3 decreased the TNF concentration that reduces cell density by 50% (ED_{50}) for TNF dose-dependently (Fig. 3). When 20:5n-3 increased from 0 to 50 μmol/L, the ED_{50} for TNF decreased tenfold, from 25 ng/L to 2.5 ng/L. A similar dose-response relationship was obtained by preincubating with increasing concentrations of 20:4n-6 or 22:6n-3 (results not shown). The absolute effect of these fatty acids changed relatively little with increasing concentrations of TNF, but was most pronounced at TNF concentrations around ED_{50}. The relative effect, however, varied with the concentration of TNF. At 0.04 ng/L TNF, 50 μmol/L 20:5n-3 reduced cell survival by 15%, while it was reduced by 65% compared to the control at 40 ng/L TNF.

Preincubation of WEHI clone 13 cells with 50 μmol/L of 20:4n-6 or 22:6n-3 decreased the ED_{50} for TNF similarly to 20:5n-3 (Fig. 4). In WEHI clone 13 cells, 50 μmol/L 22:6n-3 decreased the ED_{50} for TNF from 25 ng/L to 2.5 ng/L, while 20:4n-6 and 20:5n-3 both decreased ED_{50} to 1 ng/L (Fig. 4). In the less TNF-sensitive parental WEHI 164 cell line, 22:6n-3 decreased ED_{50} five-fold from 2.2 μg/L to 0.43 μg/L, while 20:4n-6 and 20:5n-3 at

TABLE 3

Fatty Acid Composition of the Cell Culture Medium[a]

	Phospholipids	Neutral lipids	FFA[b]
14:0	0.2 ± 0.04	22.3 ± 2.1	0.99 ± 0.08
16:0	14.8 ± 0.38	86.4 ± 17.4	6.49 ± 0.16
18:0	16.9 ± 0.45	43.8 ± 9.7	10.88 ± 0.43
20:0	0.5 ± 0.09	2.1 ± 1.0	0.12 ± 0.01
16:1n-9	0.5 ± 0.20	75.3 ± 14.1	0.89 ± 0.20
18:1n-9/7	15.3 ± 0.28	482.1 ± 105.5	5.57 ± 0.04
20:1n-9/11	0.5 ± 0.18	17.2 ± 2.5	0.13 ± 0.03
22:1n-9/11	ND[c]	ND	0.01 ± 0.01
20:3n-9	0.4 ± 0.04	4.0 ± 0.9	0.09 ± 0.01
18:3n-3	ND	13.9 ± 1.5	0.12 ± 0.01
20:5n-3	0.4 ± 0.11	27.8 ± 5.1	0.36 ± 0.005
22:5n-3	5.3 ± 0.41	14.4 ± 3.0	0.70 ± 0.01
22:6n-3	5.4 ± 0.45	34.3 ± 6.2	1.16 ± 0.01
18:2n-6	1.2 ± 0.12	117.5 ± 23.6	1.11 ± 0.01
18:3n-6	ND	3.5 ± 0.7	0.03 ± 0.002
20:2n-6	0.1 ± 0.13	0.5 ± 0.4	0.05 ± 0.01
20:3n-6	2.4 ± 0.15	20.2 ± 4.9	0.42 ± 0.006
20:4n-6	4.7 ± 0.29	123.4 ± 24.7	1.77 ± 0.002
22:4n-6	0.3 ± 0.27	1.7 ± 0.4	0.06 ± 0.006
22:5n-6	0.2 ± 0.13	6.0 ± 10.3	0.01 ± 0.02
Total fatty acids[d]	74.9 ± 5.21	1101 ± 238	31.0 ± 1.10

[a]The culture medium consisted of RPMI 1640 containing 10% (v/v) heat-inactivated fetal calf serum and 2 mmol/L L-glutamine. Results are expressed as μmol fatty acid/L culture medium, and given as mean ± SD of triplicate determinations.

[b]Free fatty acids.

[c]Not detected.

[d]Total FA includes some minor fatty acids not listed.

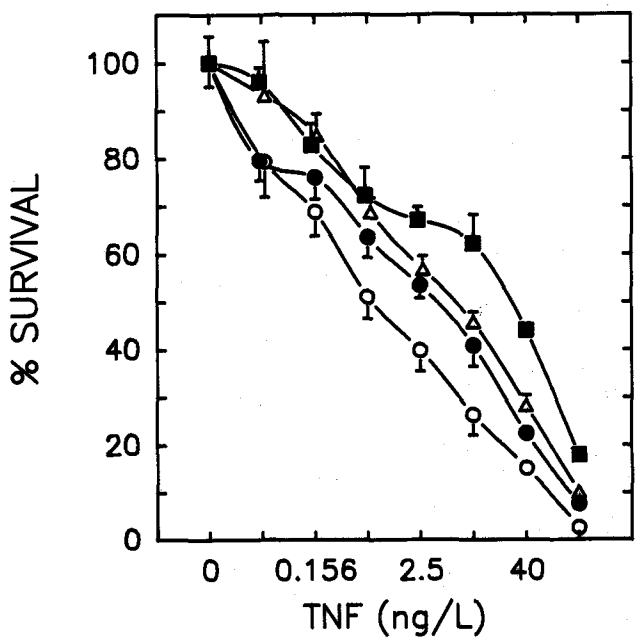

FIG. 3. Effect of increasing concentrations of 20:5n-3 on the ED_{50} for TNF in WEHI clone 13 cells. Cells were preincubated with the indicated concentrations of 20:5n-3 for 44 hr. Thereafter, the medium was removed and TNF was added in four-fold dilutions as indicated. Cell survival was measured by the MTT assay 22 hr later. In wells without TNF, the mean optical density was 1.13, 1.18, 1.14 and 1.07 after preincubation with control, 12.5, 25 and 50 μmol/L 20:5n-3, respectively. Results are from one of two similar experiments, and given as mean ± SD of quadruplicates. No addition (■), 12.5 μmol/L (△), 25 μmol/L (●), 50 μmol/L (○).

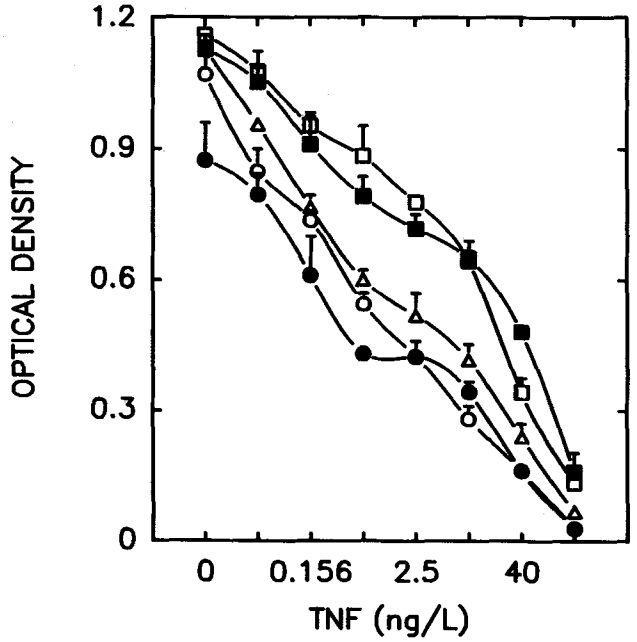

FIG. 4. Effect of enriching WEHI clone 13 cells with different fatty acids on the sensitivity to TNF. WEHI clone 13 cells were preincubated in microplates for 44 hr with or without the indicated fatty acid (50 μmol/L) supplementation, before changing to medium containing TNF in four-fold dilutions. Cell survival was measured by the MTT assay as described in Materials and Methods. Results are expressed as optical density and are given as mean ± SD of quadruplicates. Cells were preincubated with no addition (■), 18:1n-9 (□), 20:4n-6 (△), 20:5n-3 (○) or 22:6n-3 (●).

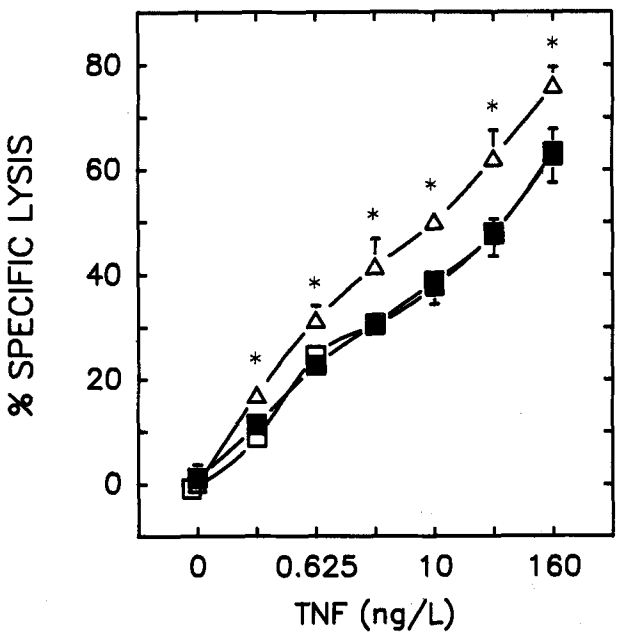

FIG. 5. TNF-induced cytolysis of WEHI clone 13 cells as measured using a 16 hr ^{51}Cr release assay as described in Materials and Methods. Cells were preincubated for 44 hr with no addition (■), 50 μmol/L 18:1n-9 (□) or 50 μmol/L 20:4n-6 (△). The spontaneous release in control, 18:1n-9- and 20:4n-6-treated cells was 23%, 24% and 27% of total release, respectively. Results are expressed as mean ± SD, and are from two experiments, each performed in triplicate. *$P < 0.05$ as compared to control. Data were analyzed by one-way analysis of variance (Scheffe F-test).

50 μmol/L decreased ED_{50} to 0.7 and 0.6 μg/L, respectively (results not shown). In Figure 4 the results are presented as optical density instead of percent survival to show that some of the fatty acids slightly decreased cell density. To further confirm that varying cell density did not invalidate the results, control experiments were performed with a constant cell density per well. Again, both 20:4n-6 and 22:6n-3 increased the sensitivity to TNF, while 18:1n-9 had no effect (results not shown), indicating that varying cell densities did not explain the effect on ED_{50}.

When the change in TNF sensitivity was measured using a 16-hr chromium release assay, 20:4n-6 decreased ED_{50} for TNF from approx. 57 ng/L to 10 ng/L, while 18:1n-9 had no effect (Fig. 5), which is similar to the results obtained using the MTT assay.

To test whether the increased sensitivity to TNF was connected with eicosanoid synthesis or free radical formation, the effect of enzyme inhibitors and antioxidants was examined (Table 4). Dexamethasone, but not indomethacin, decreased the cytotoxic effect of TNF in both fatty acid-enriched and in non-enriched cells. Further, the thromboxane synthetase inhibitor carboxyheptylimidazole had no effect. The relatively specific 5-lipoxygenase inhibitor NDGA did not inhibit TNF-induced cytotoxicity and was itself slightly toxic at 25 μmol/L when tested in the absence of TNF (results not shown). The water-soluble antioxidant ascorbic acid had no effect on TNF-induced toxicity (results not shown). The lipid soluble antioxidant BHT did not inhibit TNF-induced cytotoxicity and was toxic itself at 100 μM. In comparison, the antioxidant BHA (100 μM) totally inhibited TNF-induced cytotoxicity in both control cells as well as in cells enriched with fatty acids. This suggests that BHA does not block TNF-induced cytotoxicity through its antioxidant effect.

DISCUSSION

The present study shows that the sensitivity to TNF-induced cytotoxicity in WEHI 164 fibrosarcoma cells can be modified by preincubating the cells with different polyunsaturated fatty acids. Although polyunsaturated fatty acids inhibited the growth of WEHI cells slightly also in the absence of TNF, this could not explain the observed, increased sensitivity to TNF. Preincubation with polyunsaturated fatty acids could increase the sensitivity to TNF through several mechanisms, as discussed below.

Yamauchi *et al.* (24) showed that TNF stimulated the production of hydroxyl radicals in the TNF-sensitive U-937 cells, indicating that lipid peroxidation may be involved in TNF-mediated cytotoxicity. The observed increase in sensitivity to TNF in WEHI cells preincubated with 18:3n-6, 20:3n-6, 20:4n-6, 20:5n-3 or 22:6n-3, could therefore reflect an increased unsaturation of the cell membranes, thereby rendering the cells more susceptible to peroxidation from TNF-induced radical formation. This report indicates, however, that preincubation with polyunsaturated fatty acids selectively increases TNF-induced

TABLE 4

Effect of Inhibitors of Arachidonate Metabolism on TNF-Mediated Cytotoxicity

Additions	(μmol/L)	Fatty acid present during preincubation[a]			
		None	18:1n-9	20:4n-6	20:5n-3
		Percent of control			
Control		100.0 ± 3.6	94.0 ± 6.4	117.8 ± 6.1[b]	119.6 ± 10.9[b]
Dexamethasone	1	68.5 ± 15.4[c]	63.3 ± 16.3[c]	76.3 ± 1.9[c]	76.6 ± 7.3[c]
	10	51.5 ± 20.9[c]	47.0 ± 21.1[c]	63.8 ± 9.3[c]	68.3 ± 4.5[c]
Indomethacin	50	111.9 ± 3.1	105.1 ± 9.7	113.6 ± 17.0	120.5 ± 23.9
	100	111.7 ± 10.6	103.6 ± 23.2	103.2 ± 24.6	108.3 ± 24.6
NDGA	10	116.2 ± 10.9	107.9 ± 17.4	121.8 ± 26.0	119.4 ± 24.7
	25	124.9 ± 21.4[c]	127.1 ± 26.8[c]	138.5 ± 33.7[c]	137.1 ± 39.8
BHA	25	50.7 ± 5.7[c]	43.7 ± 4.4[c]	57.0 ± 6.9[c]	55.8 ± 6.3[c]
	100	− 2.3 ± 5.0[c]	− 6.0 ± 4.2[c]	− 0.8 ± 6.9[c]	0.8 ± 2.6[c]
BHT	25	100.8 ± 7.6	101.6 ± 6.1	118.8 ± 11.1	120.2 ± 9.2
	100	142.5 ± 30.2[c]	131.4 ± 19.9[c]	145.0 ± 28.4[c]	159.0 ± 44.7[c]
Carboxyheptylimidazole	25	106.6 ± 7.7	97.8 ± 10.2	116.7 ± 13.3	118.1 ± 9.0
	100	97.3 ± 6.4	85.1 ± 19.6	116.6 ± 9.3	114.1 ± 10.6

[a] WEHI clone 13 cells were preincubated with 50 μmol/L of the indicated fatty acid before labeling with ^{51}Cr as described in Materials and Methods. The cells were then challenged with TNF (40 ng/L) in the presence of the compounds indicated 16 hr before measuring specific cell lysis. The results are from two independent experiments and are given as mean ± SD of six determinations. The spontaneous release varied between 21 and 26% of total.

[b] $P < 0.05$ when compared to cells preincubated with ethanol only. Data were analyzed by one-way analysis of variance using Duncan multiple comparison test.

[c] $P < 0.05$ when compared to the control in each fatty acid treatment. Data were analyzed by one-way analysis of variance using Duncan multiple comparison test.

cytotoxicity in WEHI cells, and that this increase probably does not reflect merely the increased unsaturation of the cellular lipids.

Preincubating the WEHI cells with 50 μmol/L 18:3n-3 had negligible effects on TNF-induced cytotoxicity, while 18:3n-6 and 20:3n-6 reduced cell survival by 60 and 98%, respectively (Fig. 1). The trienoic fatty acid 20:3n-6 was thus considerably more effective in potentiating TNF cytotoxicity than the more unsaturated fatty acids 20:5n-3 and 22:6n-3, which reduced cell survival by 65 and 72%, respectively, at the same concentration. These results indicate that there is no direct correlation between the degree of unsaturation of the fatty acid added to the cells, and the effect on TNF-induced cytotoxicity.

Preincubation with 18:2n-6 had only small effects on the sensitivity to TNF even after 72-hr preincubation (Fig. 2). We speculate that the small effect of 18:2n-6 could be explained by a low activity of the Δ6 desaturase, the rate-limiting enzyme in the conversion of 18:2n-6 to 18:3n-6, 20:3n-6 and 20:4n-6 (25). The fatty acid analysis indicated that preincubation with 18:2n-6 did not increase 18:3n-6 and 20:4n-6 in total phospholipids. By comparison, preincubation with 18:3n-6 increased both 20:3n-6 and 20:4n-6 in total phospholipids. However, the increased sensitivity to TNF was not related only to 20:3n-6 or 20:4n-6, since preincubation with 20:5n-3 decreased both 20:3n-6 and 20:4n-6 in total phospholipids and simultaneously increased the sensitivity to TNF. The fatty acid analysis also showed that preincubation with 18:1n-9, 18:2n-6, 18:3n-3 and 18:3n-6 increased cellular 20:1n-9, 20:2n-6, 20:3n-3 and 20:3n-6, respectively, most likely due to chain elongation.

Incubation of WEHI 164 clone 13 cells with 20:5n-3 or 20:4n-6 increased the sensitivity to TNF dose- and time-dependently. Hepburn *et al.* previously reported that addition of 20:4n-6 simultaneously with TNF had no effect on TNF-induced cytotoxicity. We found that adding 20:4n-6 for 22 hr simultaneously with TNF reduced cell survival by only 14%. In comparison, preincubating the cells with 20:4n-6 for 24 hr before challenging the cells with TNF in the absence of 20:4n-6 reduced cell survival by 30% (Fig. 2). This may suggest that the increased sensitivity to TNF is not mediated by free unesterified 20:4n-6 present in the culture medium or intracellularly, and we therefore speculate that 20:4n-6 has to be incorporated into the cellular lipids in order to increase the susceptibility to TNF. A similar conclusion was recently published by Reid *et al.* (26) who showed that an adipogenic cell line was more sensitive to TNF after enrichment of choline- and ethanolamine-containing phospholipids with 20:4n-6.

The fatty acids were not present simultaneously with TNF in most of our experiments. Therefore we possibly underestimated the fatty acid effects since simultaneous addition of 20:4n-6 and TNF increased TNF toxicity slightly. Preincubation of WEHI clone 13 cells with 50 μmol/L 20:4n-6 in microplates decreased the ED_{50} for TNF to approximately 4% of control cells (Fig. 4). However, preincubation with 50 μmol/L 20:4n-6 in the petri dishes in the ^{51}Cr release experiments decreased the ED_{50} for TNF to approximately 18% of control cells (Fig. 5). The fatty acid concentration was identical in both types of experiments, but due to differences in cell counts, only 1 pmol fatty acid was added per cell in the ^{51}Cr

release experiments compared to 7.5 pmol/cell in the microplate experiments. This may explain at least some of the difference.

The effect of polyunsaturated fatty acids on TNF-induced cytotoxicity in WEHI cells may possibly be mediated by free radicals, and we therefore tested the effect of antioxidants. Other investigators have shown that incubation with 20:3n-6, 20:4n-6, 20:5n-3 and 22:6n-3 may increase free radical generation (27), inhibit growth, and even kill some cancer cell lines *in vitro* (28,29). Further, it has been reported previously that some, but not all, free radical scavengers can partly inhibit TNF-induced cytotoxicity (30,31). In the present study, the antioxidant BHA completely blocked TNF-induced cytotoxicity in both control as well as in fatty acid-enriched cells (Table 4), while the structurally and functionally very similar antioxidant BHT did not inhibit TNF-induced cytotoxicity. The results suggest that BHA does not block TNF-induced cytotoxicity in WEHI cells through its antioxidant effect, but through some other unknown mechanism. We therefore speculate that BHA functions as a specific enzyme inhibitor.

Previous reports have suggested that TNF-induced cytotoxicity is linked to arachidonic acid metabolism (10), and we therefore tested several other enzyme inhibitors. BHA has been reported to inhibit both the lipoxygenase enzyme(s) (32) and cyclooxygenase (33). However, since the relatively specific 5-lipoxygenase inhibitor NDGA had no effect, while in comparison the antioxidant and lipoxygenase inhibitor BHA completely blocked TNF-induced toxicity, this may suggest that if a lipoxygenase is involved in TNF-induced toxicity, it may be different from the 5-lipoxygenase. It is unlikely that BHA blocks TNF-induced cytotoxicity due to inhibition of the cyclooxygenase, since indomethacin did not inhibit TNF cytotoxicity in these cells. The lack of effect of carboxyheptylimidazole suggests that thromboxane synthetase metabolites are not involved in TNF-mediated cytotoxicity in WEHI clone 13 cells. The inhibitory effect of dexamethasone on TNF-mediated cytotoxicity is in agreement with the report by Kull (9). The effect of dexamethasone may result from inhibited phospholipase A_2 activity as indicated by other reports (11), and/or from lowering the affinity for TNF to its receptor (9).

The present report is in agreement with the report by Hori *et al.* (34) who showed that TNF-insensitive diploid FS-4 fibroblasts became sensitive to the cytotoxic effect of TNF in the presence of 20:4n-6 or 20:5n-3. They also showed that the cytotoxic effect of TNF in the presence of 20:4n-6 was completely abolished by indomethacin. As indomethacin had no effect on TNF-induced cytotoxicity in WEHI cells, this may suggest that TNF-induced cytotoxicity in diploid fibroblasts and WEHI fibrosarcoma cells involves different mechanisms.

In conclusion, our report indicates that the sensitivity to TNF in WEHI 164 fibrosarcoma cells can be increased selectively by preincubation with long-chain polyunsaturated fatty acids. The results suggest that the increased sensitivity to TNF is not only due to an increased unsaturation of cell membranes. Further, free 20:4n-6 seems to be less potent than 20:4n-6 incorporated into cell lipids. Future studies should clarify if it is the fatty acids themselves, some fatty acid metabolites, or other effects of fatty acids that increase the sensitivity to TNF-induced cytotoxicity. Further investigations are also needed to evaluate if a diet enriched in such fatty acids in any way is associated with resistance against cancer *in vivo*.

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The Effect of Dietary Supplementation with Eicosapentaenoic Acid on the Phospholipid and Fatty Acid Composition of Erythrocytes of Marmoset

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Adult male marmoset monkeys were fed eicosapentaenoic acid (20:5n-3) as the ethyl ester in diets containing either 32% (reference diet, no added cholesterol) or 7% (atherogenic diet with 0.2% added cholesterol) linoleic acid (18:2n-6) for 30 wk. No changes were seen in the level of phosphatidylcholine (PC) or phosphatidylethanolamine (PE) but minor changes were observed in both the sphingomyelin (SPM) and phosphatidylinositol plus phosphatidylserine (PI + PS) fractions of erythrocyte lipids. The extent of total n-3 fatty acid incorporation into membrane lipids was higher in atherogenic diets (polyunsaturated/monounsaturated/saturated (P/M/S) ratio 0.2:0.6:1.0) than reference diets (P/M/S ratio 1:1:1) and this was true for both PE (33.4 ± 1.0% *vs* 24.3 ± 1.1%) and PC (9.3 ± 0.5% *vs* 4.9 ± 0.3%). Although suitable controls for cholesterol effects were not included in the study, earlier results obtained with marmosets lead us to believe such effects were probably small. Regardless of basic diet (atherogenic, reference), 20:5n-3 was preferentially incorporated into PE (10.8 ± 0.2%, 6.0 ± 0.02%) while smaller amounts were incorporated into PC (6.9 ± 0.4%, 3.2 ± 0.2%). The major n-3 polyunsaturated fatty acid found in PE in response to dietary 20:5n-3 was the elongation metabolite 22:5n-3 in both the atherogenic (17.7 ± 0.7%) and reference (14.3 ± 1.0%) dietary groups; 22:6n-3 levels were less affected by diet (4.7 ± 0.3% and 3.9 ± 0.2%, respectively). The results can be interpreted to indicate an inverse relationship between the amount of dietary 18:2n-6 and incorporation of 20:5n-3 into erythrocyte membrane phospholipids regardless of whether the major dietary n-3 fatty acid was α-linolenate (18:3n-3) or 20:5n-3. This interpretation is supported by theoretical calculations.

Lipids 27, 169–176 (1992).

The n-3 polyunsaturated fatty acid eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) which are present in marine oils have unique lipid lowering potential in humans and animals (1,2). Fish oil has been shown to lower triglyceride levels in both normal and hypertriglyceridemic subjects (3). In the rat, the hypotriglyceridemic effect is probably mediated by 20:5n-3 while 22:6n-3 may be responsible for lowering of plasma cholesterol levels (4). Although investigations into the effect of n-3 polyunsaturated fatty acids on blood cells have tended to focus on platelets (5,6) or neutrophils (7,8), changes in phospholipid fatty acids and membrane function of erythrocytes have also been examined. For example, dietary n-3 fatty acids have been shown to affect both the deformability of red cells (9,10) and the fluidity of erythrocyte membranes from human subjects (11,12). Similar studies on rats have failed to demonstrate changes in erythrocyte deformability despite an increase in n-3 polyunsaturated fatty acids in membrane phospholipids (13).

Many beneficial effects of marine oil consumption by humans are now attributed to incorporation of 20:5n-3 into cell membranes (1,2). However, because 20:5n-3 incorporation into liver phospholipids has been shown to be less when fish oil was fed with safflower oil than when it was fed with beef tallow (14), co-ingestion of other fats, particularly those rich in linoleate (18:2n-6) must be considered in order to maximize 20:5n-3 incorporation.

Interpretation of results from animals fed marine oils has often been difficult due to the presence of several n-3 polyunsaturated fatty acids in these oils. The availability of a source of ethyl esters derived from fish oil which contained over 70% 20:5n-3 and no 22:6n-3, made it possible to determine the effect of dietary 20:5n-3 on the fatty acid composition of individual phospholipids of the marmoset red blood cell in the absence of other n-3 polyunsaturated fatty acids. We demonstrate that dietary 20:5n-3 is preferentially incorporated into erythrocyte phosphatidylethanolamine (PE), and this incorporation is greatest when dietary 18:2n-6 is reduced as in an atherogenic-type diet.

MATERIALS AND METHODS

Marmosets. Adolescent male common cotton-eared marmosets (*Callithrix jacchus jacchus*), approximately 16 to 26 mon old at the start of the experiment, were divided into four groups of equivalent age and weight. Marmosets were paired for optimum growth and social behavior and kept in aluminum alloy marmoset cages in a room with fluorescent light and 30 min of ultraviolet irradiation daily. The temperature was maintained at 26°C and the humidity at 50%. Marmosets were maintained on the various dietary lipid regimes described below for a period of 30 wk and were sacrificed under anaesthesia by injection of Saffan, 1.5 mL per kg body weight (alphaxalone, 9 mg/mL; alphadolone acetate, 3 mg/mL; Glaxo, Sydney, Australia), into the femoral artery.

Marmoset diets. Four high-fat dietary regimes of equivalent energy value were used. These diets were chosen to examine the effect of 20:5n-3 (as the ethyl ester) when administered in combination with diets differing significantly in their polyunsaturated/monounsaturated/saturated (P/M/S) fatty acid ratio. The normal colony diet for marmosets consisted of a 1:1 mixture of greyhound chow (Arnott Harper's Ltd., Adelaide, Australia) and primate meal (Milling Industries Ltd., Adelaide, Australia). The overall composition of this diet with regard to all nutrients has previously been described (15), and contained approximately 4.5% fat. The fatty acid composition of the normal colony diet (shown in Table 1) was used as the basis

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Abbreviations: ATH, atherogenic diet; EPA, eicosapentaenoic acid; FID, flame-ionization detector; HUFA, highly unsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; P/M/S, polyunsaturated/monounsaturated/saturated ratio; REF, reference diet; SPM, sphingomyelin.

for the preparation of the four experimental diets described below.

The reference (REF) diet consisted of the colony diet supplemented with 5.5% (by weight) sunflower seed oil (Nuttelex, Melbourne, Australia), 1.5% olive oil (SA Olive Oil Co., Adelaide, Australia) and 3.0% sheep kidney (perirenal) fat (Noarlunga Meat Works, Noarlunga, Australia). The final fat blend achieved a P/M/S ratio of 1.1:1.1:1.0. Addition of 0.8 (w/w) 20:5n-3 ethyl ester concentrate (containing 75% 20:5n-3 and 0.2% α-tocopherol; supplied by Dr. Yasushi Tamura, Chiba University Medical School, Chiba, Japan) changed the P/M/S ratio to 1.1:1.0:1.0 (REF + eicosapentaenoic acid (EPA) diet). The atherogenic (ATH) diet consisted of the colony diet supplemented with 10% sheep kidney fat and 0.2% (w/w) cholesterol (Ajax Chemical Co., Adelaide, Australia) which achieved a P/M/S ratio of 0.14:0.6:1.0. The addition of 0.8% (w/w) 20:5n-3, ethyl ester concentrate to the ATH diet gave a P/M/S ratio of 0.2:0.6:1.0 (ATH + EPA diet). The fatty acid compositions of the 20:5n-3 concentrate and of the experimental diets are shown in Table 1. All additions (*i.e.* oils, fat, cholesterol and concentrate) were thoroughly mixed with the crushed colony diet before the respective diets were repelleted. A two-week supply of each diet was prepared and frozen at −20°C in a N_2 atmosphere. Aliquots of these diets were thawed for each daily feeding and replaced daily. Animals were fed *ad libitum*.

Collection of erythrocytes. Immediately after the animals were killed, blood was collected from the aorta by a heparinized syringe, and centrifuged to separate plasma and erythrocytes as previously described (16). Erythrocytes were washed three times by centrifugation in phosphate buffered saline, and lipids were extracted immediately.

Lipid extraction. Lipids were extracted by the methods described elsewhere (16). Briefly, one volume of washed erythrocytes was extracted with 4 vol of boiling 2-propanol containing the antioxidant butylated hydroxyanisole (0.1% of the estimated lipid weight), and the mixture was boiled for 30 sec. After cooling, 8 vol of chloroform and 2 vol of water were added, and the mixture was shaken. Following centrifugation, the lower organic phase was collected and the aqueous phase was re-extracted with an additional 4 vol of chloroform. The combined organic phases were dried over anhydrous sodium sulfate.

Separation of total phospholipids and phospholipid classes. Total phospholipids were separated from neutral lipids by thin-layer chromatography on silica gel H plates using petroleum hydrocarbon acetone (3:1, v/v) as the developing solvent. Individual phospholipid classes (as % of total phospholipids) were separated on Whatman LK5D thin-layer chromatography plates (Maidstone, England) developed in one direction in a solvent system of chloroform/ethanol/water/triethylamine (30:34:8:35, by vol) as described by Touchstone *et al.* (17). All phospholipids were clearly separated except phosphatidylinositol (PI) and phosphatidylserine (PS) which were only present in trace amounts; samples were pooled as indicated in Table 2.

Fatty acid analysis of erythrocyte total phospholipids and phospholipid classes. Phospholipid bands isolated by thin-layer chromatography were scraped directly into glass vials containing 1% (v/v) H_2SO_4 in methanol and heated for 3 hr at 70°C. The resulting methyl esters were extracted into heptane and dried over sodium sulfate prior to analysis by gas chromatography. Analyses of fatty acid methyl esters of the total phospholipids and the major phospholipid classes of marmoset erythrocytes were performed using capillary gas chromatography (Hewlett-Packard HP 5880 gas chromatograph; Hewlett-Packard, Palo Alto, CA). The column was a 50-m glass column

TABLE 1

Fatty Acid Composition of Marmoset Colony Diet, 20:5n-3(EPA) Concentrate and Experimental Diets[a]

Major fatty acid (%; w/w)	Colony diet	Concentrate	Experimental diets			
			REF	REF + EPA	ATH	ATH + EPA
Σ Saturated	46.0	2.1	31.3	31.8	56.1	55.9
Σ Monounsaturated	39.5	16.2	33.7	32.9	35.9	32.7
n-6						
18:2	13.1	0.6	32.9	31.0	6.9	6.6
20:4	tr.	4.5	n.d.	tr.	n.d.	tr.
n-3						
18:3	1.4	0.5	0.9	0.9	1.1	1.0
20:5	n.d.	74.8	n.d.	2.9	n.d.	3.3
Σ(n-6)	13.1	5.1	32.9	31.0	6.9	6.6
Σ(n-3)	1.4	75.3	0.9	3.8	1.1	4.3
Σ(n-6)/Σ(n-3)	9.4	0.07	36.5	8.2	6.3	1.5
U.I.	70	411	102	112	53	65
Cholesterol(%)	<0.04	n.d.	<0.04	<0.04	0.2	0.2
Total fat(%)	4.5	—	14.3	14.3	14.8	15.3
Energy Value(KJ/g)			22.9	22.8	22.8	22.7

[a]Fatty acids are designated by the number of carbon atoms followed by the number of double bonds. The particular unsaturated fatty acid series is shown as (n-x) which refers to the first double bond counting from the terminal methyl group of the fatty acid. Fatty acid values shown above represent the average obtained from at least five separate extractions and analyses of the respective diet or oil. tr, (trace), present at less than 0.5%; n.d., not detected. REF, reference; EPA, eicosapentaenoate; ATH, atherogenic; ATH + EPA, atherogenic plus eicosapentaenoate; U.I., unsaturation index, as defined in reference (24).

coated with SP2340 (Supelco Inc., Bellefonte, PA) prepared by Chromolytic Technology Ltd. (Boronia, Victoria, Australia). Methyl esters were separated using helium as carrier gas at a flow of 25 cm/sec with a temperature gradient of 120°C to 200°C at 5°C per minute. The injection/split temperature was 250°C and the flame-ionization detector (FID) temperature was 300°C. Fatty acids were identified using authentic lipid standards supplied by Nuchek Prep (Elysian, MN).

Quantitative diet-tissue relationships. It has been reported (18,19) that the amounts of certain 20 and 22 carbon n-3, n-6 and n-9 highly unsaturated fatty acids (HUFA) that are maintained in membrane phospholipids fit saturable hyperbolic relationships to the dietary supply of precursors and can be described by the following equations (Equation 1, 2 and 3):

$$\text{n-6 as \% HUFA} = \frac{100}{1 + \frac{HC6}{en\%H6}\left(1+\frac{en\%H3}{HC3}\right)} + \frac{100}{1 + \frac{PC6}{en\%P6}\left(1 + \frac{en\%P3}{PC3} + \frac{en\%H3}{HI3} + \frac{en\%O}{Co} + \frac{en\%P6}{Ks}\right)} \quad [1]$$

$$\text{n-3 as \% HUFA} = \frac{100}{1 + \frac{HC3}{en\%H3}\left(1+\frac{en\%H6}{HC6}\right)} + \frac{100}{1 + \frac{PC3}{en\%P3}\left(1 + \frac{en\%P6}{PC6} + \frac{en\%H6}{HI6} + \frac{en\%O}{Co} + \frac{en\%P3}{Ks}\right)} \quad [2]$$

$$\text{n-9 as \% HUFA} = \frac{100}{1 + K\left(1 + \frac{en\%P6}{PC6} + \frac{en\%H6}{HI6} + \frac{en\%P3}{PC3} + \frac{en\%H3}{HI3}\right)} \quad [3]$$

The variables are: en%P6 = energy % as 18:2n-6; en%P3 = energy % as 18:3n-3; en%H6 = energy % as 20:4n-6; and en%H3 = energy % as 20:5n-3. The fitted constants are: PC3 = 0.0685; HC3 = 1.0; HI3 = 0.0115; PC6 = 0.17; HC6 = 0.8; HI6 = 0.02; Co = 10; Ks = 0.17; and K = 3.9. The equation predicts the level of certain n-6 (20:3, 20:4), n-3 (20:5, 22:5) and n-9 (20:3) HUFA in tissues based on the level of dietary fatty acids including these same HUFA as well as the level of dietary 18:2n-6 and 18:3n-3. Note that 22:6n-3, a major constituent of some tissues, is not predicted by the equations.

Statistical analysis. All data are presented as the mean ± standard error of the mean for the indicated number of samples (n) for each dietary group. Multiple comparisons were made between dietary groups by unpaired t-tests with $p < 0.05$ being considered significant.

Chemicals. Solvents were of the highest analytical grade and were redistilled and gassed with N_2 before use. All other chemicals were of the highest reagent grade available.

RESULTS

By mixing the various fats and oils in the proportions indicated in Materials and Methods, two high-fat diets of equal energy value were produced which differed markedly in their P/M/S ratio and hence in their proportion of 18:2n-6 without affecting the level of α-linolenate (18:3n-3). Addition of the 20:5n-3 concentrate to these diets resulted in about 3% of the total dietary fatty acids available as 20:5n-3 (Table 1). Body weights of marmosets (g) at the start and finish of the experiment for each dietary group were (mean ± SEM): REF (N = 8) 353 ± 7, 364 ± 10; REF + EPA (n = 8) 332 ± 9, 326 ± 9; ATH (n = 10) 324 ± 8, 335 ± 12; ATH + EPA (n = 10) 313 ± 9, 307 ± 12.

The proportions of marmoset erythrocyte phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were not altered by the dietary supplements. PC remained at about 40% of the total erythrocyte phospholipids, while PE was about 32% (mean values) (Table 2).

The proportion of sphingomyelin (SPM) was reduced in all dietary groups in comparison to the REF diet; however this difference was only statistically significant when compared to the ATH + EPA group. In comparison to the REF group, the proportion of PI + PS was significantly elevated in the three other dietary groups with the proportion being about double that of the control. The increase in phosphatidylinositol plus phosphatidylserine (PI + PS) therefore appeared to be at the expense of SPM.

Eicosapentaenoic acid (20:5n-3) was significantly incorporated into the total phospholipids of erythrocyte membranes in those animals receiving 20:5n-3 supplements (Table 3). Interestingly the incorporation of 20:5n-3 in the ATH + EPA group (10%) was approximately double that of the REF + EPA group (5.4%). Accompanying this increase in the proportion of 20:5n-3 was an increase in the proportion of its elongation product docosapentaenoic acid (22:5n-3) but its Δ4 desaturase metabolite, 22:6n-3, did not increase.

In the REF + EPA group, the proportion of 22:5n-3 actually exceeded that of 20:5n-3 (Table 3). Together these changes resulted in a marked increase in the proportion of the total n-3 polyunsaturated fatty acids in both 20:5n-3 supplemented groups. Also accompanying the increase in 20:5n-3 was a concomitant decrease in the proportions of 18:2n-6 and arachidonic acid (20:4n-6) in the REF + EPA and ATH + EPA diet groups. The combined decrease in these two n-6 polyunsaturated fatty acids approximated the increase in the proportion of 20:5n-3 with 20:5n-3 supplementation. As a result, the n-6/n-3 polyunsaturated fatty acid ratio markedly decreased in the two EPA-supplemented diet groups.

TABLE 2

Effect of Dietary Lipid Supplementation on the Proportion of Major Phospholipids of Marmoset Erythrocytes[a]

Phospholipid class	REF[b] (n = 8)	REF + EPA (n = 8)	ATH (n = 10)	ATH + EPA (n = 10)
PC	41.8 ± 1.1	40.8 ± 1.6	39.6 ± 0.9	40.1 ± 0.8
PE	31.5 ± 0.7	31.8 ± 1.4	31.8 ± 1.1	32.5 ± 0.9
SPM	23.1 ± 1.4	19.7 ± 1.4	20.8 ± 1.8	20.1 ± 0.8
PI + PS	3.6 ± 1.1[c]	7.7 ± 1.5	7.8 ± 0.9	7.3 ± 1.1

[a]Data are presented as the mean ± SEM for the indicated number of animals (n) in each dietary group. Values for PI + PS were determined from 2 pooled samples per diet group each representing 4 individual animals.

[b]% of total phospholipids.

[c]Significantly different from the other three dietary groups at $P < 0.05$.

TABLE 3

Effect of Dietary Lipid Supplementation on the Fatty Acid Composition of the Total Phospholipids of Marmoset Erythrocytes[a]

Major acid (1%; w/w)	REF (n = 6)	REF + EPA (n = 8)	ATH (n = 10)	ATH + EPA (n = 10)
Σ Saturated	38.7 ± 0.1[b]	40.7 ± 0.3[c]	39.7 ± 0.3[d]	42.2 ± 0.3[e]
Σ Monounsaturated	19.4 ± 0.1[b]	18.9 ± 0.3[b]	23.1 ± 0.4[c]	21.2 ± 0.4[d]
n-6				
18:2	21.0 ± 0.6[b]	15.7 ± 0.3[c]	13.0 ± 0.2[d]	7.1 ± 0.1[e]
20:2	0.5 ± 0.01	0.30 ± 0.03	n.d.	n.d.
20:3	0.7 ± 0.1[b]	0.3 ± 0.01[c]	1.0 ± 0.07[d]	0.2 ± 0.01[e]
20:4	9.1 ± 0.2[b]	6.8 ± 0.1[c]	11.4 ± 0.1[d]	6.3 ± 0.1[e]
22:4	4.5 ± 0.2[b]	0.4 ± 0.03[c]	1.5 ± 0.1[d]	0.2 ± 0.02[e]
22:5	2.0 ± 0.1[b]	0.2 ± 0.01[c]	0.8 ± 0.04[d]	0.2 ± 0.01[e]
n-3				
18:3	0.1 ± 0.01	0.1 ± 0.01	0.6 ± 0.02	0.2 ± 0.01
20:5	0.1 ± 0.01[b]	5.4 ± 0.1[c]	0.7 ± 0.1[d]	10.0 ± 0.2[e]
22:5	1.6 ± 0.1[b]	8.8 ± 0.4[c]	4.1 ± 0.1[d]	10.6 ± 0.2[e]
22:6	2.3 ± 0.1[b]	2.9 ± 0.2[c]	3.1 ± 0.1[d]	2.9 ± 0.1[c]
Σ(n-6)	35.9 ± 0.3[b]	23.4 ± 0.2[c]	27.8 ± 0.2[d]	13.8 ± 0.2[e]
Σ(n-3)	6.2 ± 0.2[b]	17.2 ± 0.4[c]	9.3 ± 0.2[d]	23.8 ± 0.3[e]
Σ(n-6)/Σ(n-3)	5.79	1.36	2.99	0.58
U.I.	151	170	153	183

[a]Data are presented as the mean ± SEM for samples from the indicated number of animals (n) in each dietary group. Fatty acids are designated as in Table 1. Only those fatty acids present at >0.5% of the total (w/w) are reported. Values without a common superscript are significantly different at $p < 0.05$. Statistical significance was not determined for those values without a superscript.

Differences in the fatty acid composition of total phospholipids were also evident when comparing the basic diets which differed in their P/M/S ratio (Table 3). For example, relatively higher levels of 18:2n-6 and lower levels of monounsaturates were apparent in the REF and REF + EPA dietary groups (P/M/S; 1:1:1) compared to the ATH and the ATH + EPA diet groups (P/M/S; 0.2:0.6:1.0). The level of erythrocyte membrane lipid saturation was not markedly altered despite the large differences in the extent of lipid saturation between the REF- and the ATH-based experimental diets (Table 1).

Changes in the fatty acid profile of erythrocyte PE were qualitatively and quantitatively similar to those occurring in the total erythrocyte phospholipids (Table 4).

Thus the proportions of 20:5n-3, 22:5n-3 and total n-3 polyunsaturated fatty acids were increased, and the proportions of 18:2n-6, 20:4n-6, total n-6 polyunsaturated fatty acids, and the n-6/n-3 decreased with 20:5n-3 supplementation in both the REF and ATH diet groups. Accumulation of 22:5n-3 into the PE fraction was well above that of 20:5n-3 in the diets supplemented with 20:5n-3 but the level of 22:6n-3 was increased only in the REF + EPA diet relative to REF. The proportion of saturated fatty acids was not greatly influenced as a result of feeding the atherogenic diet. The changes in PC as a result of 20:5n-3 supplementation were similar in most respects to those in PE, however the extent of incorporation of 20:5n-3 was not as great (Table 5).

PC also had higher levels of saturated fatty acids, 18:2n-6 and very much less 20:4n-6. The fatty acid profile of marmoset erythrocyte SPM was characteristic of this phospholipid species in having a high proportion of saturated fatty acids, particularly 16:0, lower proportions of 18:2n-6 and 20:4n-6, and the presence of both long-chain

TABLE 4

Effect of Dietary Lipid Supplementation on the Fatty Acid Composition of Phosphatidylethanolamine of Marmoset Erythrocytes[a]

Major acid (%; w/w)	REF (n = 8)	REF + EPA (n = 8)	ATH (n = 10)	ATH + EPA (n = 10)
Σ Saturated	29.1 ± 0.4^{b}	32.3 ± 0.7^{c}	30.0 ± 0.6^{b}	32.2 ± 0.8^{c}
Σ Monounsaturated	20.6 ± 0.6^{b,c}	20.8 ± 0.8^{b,c}	22.6 ± 0.7^{b}	20.4 ± 0.6^{c}
n-6				
18:2	15.8 ± 0.6^{b}	11.4 ± 0.3^{c}	9.5 ± 0.3^{d}	4.0 ± 0.1^{e}
20:2	0.6 ± 0.01	0.3 ± 0.02	n.d.	n.d.
20:3	0.9 ± 0.1^{b}	0.3 ± 0.01^{c}	1.1 ± 0.05^{b}	0.2 ± 0.01^{d}
20:4	14.6 ± 0.5^{b}	10.0 ± 0.3^{c}	18.9 ± 0.6^{d}	9.3 ± 0.3^{c}
22:4	8.8 ± 0.3^{b}	0.7 ± 0.05^{c}	3.3 ± 0.1^{d}	0.3 ± 0.02^{e}
22:5	3.9 ± 0.2^{b}	0.4 ± 0.05^{c}	1.6 ± 0.1^{d}	0.1 ± 0.03^{e}
n-3				
20:5	0.1 ± 0.03^{b}	6.0 ± 0.1^{c}	0.8 ± 0.1^{d}	10.8 ± 0.2^{e}
22:5	2.8 ± 0.1^{b}	14.3 ± 1.0^{c}	6.4 ± 0.3^{d}	17.7 ± 0.7^{e}
22:6	2.9 ± 0.1^{b}	3.9 ± 0.2^{c}	5.0 ± 0.2^{d}	4.7 ± 0.3^{c,d}
Σ(n-6)	44.5 ± 0.4^{b}	23.1 ± 0.4^{c}	34.2 ± 0.6^{d}	13.9 ± 0.4^{e}
Σ(n-3)	5.9 ± 0.2^{b}	24.3 ± 1.1^{c}	12.5 ± 0.5^{d}	33.4 ± 1.0^{e}
Σ(n-6)/Σ(n-3)	7.60	0.95	2.74	0.42
U.I.	201	215	207	238

[a]Data are presented as the mean ± SEM for samples from the indicated number of animals (n) in each dietary group. All other data are as described in Table 1. Only those fatty acids present at >0.05% of the total (w/w) are reported. Data were analyzed as described for Table 3.

TABLE 5

Effect of Dietary Lipid Supplementation on the Fatty Acid Composition of Phosphatidylcholine of Marmoset Erythrocytes[a]

Major fatty acid (%; w/w)	REF (n = 8)	REF + EPA (n = 8)	ATH (n = 10)	ATH + EPA (n = 10)
Σ Saturated	45.7 ± 0.4^{b}	48.8 ± 0.8^{c,d}	46.3 ± 0.5^{b}	50.3 ± 0.6^{d}
Σ Monounsaturated	21.6 ± 0.4^{b}	22.8 ± 0.4^{b}	30.4 ± 0.4^{c}	27.4 ± 0.3^{d}
n-6				
18:2	27.6 ± 0.7^{b}	21.1 ± 0.5^{c}	17.7 ± 0.3^{d}	9.9 ± 0.2^{e}
20:3	0.6 ± 0.1^{b}	0.2 ± 0.01^{c}	0.9 ± 0.05^{d}	0.3 ± 0.0^{e}
20:4	2.4 ± 0.3^{b,c,d}	2.0 ± 0.1^{c}	2.6 ± 0.2^{d}	2.7 ± 0.1^{d}
n-3				
20:5	0.3 ± 0.1^{b}	3.2 ± 0.2^{c}	0.1 ± 0.01^{d}	6.9 ± 0.4^{e}
22:5	0.2 ± 0.02^{b}	1.2 ± 0.1^{c}	0.5 ± 0.04^{d}	1.7 ± 0.1^{e}
Σ(n-6)	31.9 ± 0.6^{b}	23.6 ± 0.6^{c}	21.6 ± 0.5^{d}	12.9 ± 0.3^{e}
Σ(n-3)	0.7 ± 0.1^{b}	4.9 ± 0.3^{c}	1.4 ± 0.1^{d}	9.3 ± 0.5^{e}
Σ(n-6)/Σ(n-3)	43.10	4.85	14.93	1.39
U.I.	90	95	81	101

[a]Data are presented as the mean ± SEM for samples from the indicated number of animals (n) in each dietary group. All other details are as described in Table 1. Only those fatty acids present at >0.5% of the total (w/w) are reported. Data were analyzed as described for Table 3.

saturated (20:0, 22:0; 24:0) and monoenoic (24:1n-9) fatty acid (Table 6).

Although n-3 supplementation increased the proportion of 20:5n-3, the final proportion remained at less than 1% of the total SPM fatty acids. The proportion of 18:2n-6 decreased as a result of 20:5n-3 supplementation in both diet groups. The proportion of saturated fatty acids in SPM was increased in the ATH and ATH + EPA diet groups in comparison to the REF and REF + EPA diet groups due mainly to an increase in the proportion of 18:0. Reliable measures of the constituent fatty acids in the PI + PS fractions could not be obtained due to the low levels of these phospholipids in marmoset erythrocyte membranes.

By fitting the dietary fatty acids as energy percent values into the equation devised by Lands *et al.* (18 and Lands, W.E.M., personal communication) along with the appropriate constants, it was possible to compare the levels predicted from the equations with the level of n-3, n-6 and n-9, 20 and 22 carbon HUFA observed in erythrocyte total phospholipids (Fig. 1). There was good agreement between actual and predicted n-3 HUFA for all

TABLE 6

Effect of Dietary Lipid Supplementation on the Fatty Acid Composition of Sphingomyelin of Marmoset Erythrocytes[a]

Major fatty acid (%; w/w)	REF (n = 8)	REF + EPA (n = 8)	ATH (n = 10)	ATH + EPA (n = 10)
Saturated				
14:0	0.8 ± 0.1	0.70 ± 0.1	0.9 ± 0.1	0.8 ± 0.2
16:0	33.4 ± 1.2^{b,c}	34.7 ± 0.8^{b,c}	32.1 ± 1.4^{b}	36.7 ± 1.1^{c}
17:0	0.9 ± 0.1^{b}	0.9 ± 0.1^{b}	1.2 ± 0.1^{b,d}	1.4 ± 0.1^{c,d}
18:0	14.2 ± 1.3^{b,c}	13.1 ± 0.9^{b}	17.6 ± 1.4^{c,d}	18.5 ± 1.3^{d}
20:0	1.8 ± 0.1^{b}	1.7 ± 0.1^{b}	2.4 ± 0.1^{c}	2.2 ± 0.1^{c}
22:0	8.0 ± 0.5^{b}	8.0 ± 0.6^{b}	8.2 ± 0.5^{b}	7.9 ± 0.6^{b}
24:0	8.0 ± 0.6^{b}	8.3 ± 0.7^{b}	6.8 ± 0.4^{b}	7.6 ± 0.5^{b}
Monounsaturated				
18:1n-9	12.6 ± 2.3^{b}	12.9 ± 2.3^{b}	14.1 ± 1.3^{b}	12.1 ± 1.5^{b}
24:1n-9	9.8 ± 0.6^{b}	10.3 ± 0.6^{b}	10.6 ± 0.8^{b}	7.2 ± 0.6^{c}
n-6				
18:2	5.9 ± 0.7^{b}	4.4 ± 0.5^{b}	4.6 ± 0.4^{b}	2.8 ± 0.3^{c}
20:4	0.6 ± 0.05^{b}	0.6 ± 0.01^{b}	0.8 ± 0.1^{b}	0.7 ± 0.1^{b}
n-3				
20:5	n.d.	0.4 ± 0.1	n.d.	0.7 ± 0.1
22:5	n.d.	0.6 ± 0.1	n.d.	0.7 ± 0.2
Unknown	4.2 ± 0.4^{b}	3.7 ± 0.4^{b}	0.9 ± 0.1^{c}	0.9 ± 0.1^{c}
Σ Sat	66.7 ± 1.3^{b}	67.4 ± 1.8^{b}	69.0 ± 1.7^{b}	75.1 ± 2.1^{c}
Σ Monounsaturated	22.7 ± 1.8^{b,c}	23.2 ± 1.8^{b,c}	24.6 ± 1.4^{b}	19.3 ± 2.0^{c}
Σ Polyunsaturated	10.6 ± 1.8^{b}	9.4 ± 1.8^{b}	6.4 ± 1.4^{b}	5.6 ± 2.0^{b}
Σ(n-6)	6.4 ± 0.7^{b}	5.0 ± 0.4^{b}	5.3 ± 0.4^{b}	3.5 ± 0.4^{c}
Σ(n-3)		1.0 ± 0.1		1.4 ± 0.2
Σ(n-6)/Σ(n-3)	1.51	1.16	5.78	1.88
U.I.	62	62	43	40

[a]Data are presented as the mean ± SEM for samples from the indicated number of animals (n) in each dietary group. All other data are as described in Table 1. Only those fatty acids present at >0.5% of the total (w/w) are reported. Data were analyzed as described for Table 3.

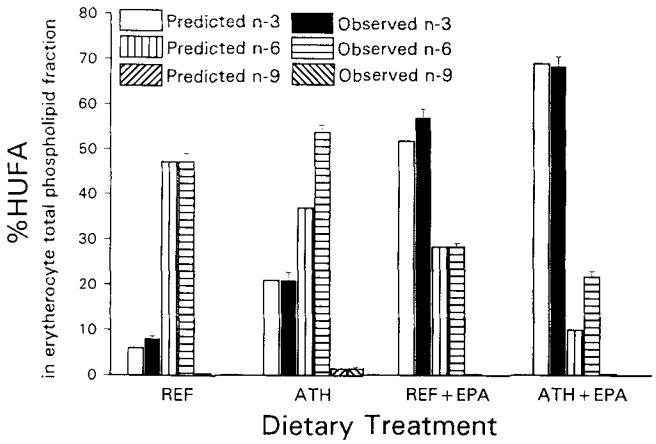

FIG. 1. Predicted levels of n-3, n-6 and n-9 highly unsaturated fatty acid (HUFA) levels in erythrocyte total phospholipids compared to actual levels observed in membrane extracts of marmosets fed different diets. Error bars indicate standard deviation of mean values.

dietary treatments. The level of n-6 HUFA was accurately predicted in both the REF and REF + EPA diets but poorly predicted in the two ATH-based diets.

DISCUSSION

This study was designed to compare the incorporation of EPA into erythrocyte membranes in two contrasting basal diets namely an atherogenic diet high in saturates containing 0.2% cholesterol and a reference diet rich in linoleate and low in cholesterol. Thus the elevated n-3 incorporation in the ATH group could be accounted for by cholesterol effects, polyunsaturate effects, or both. Without having done direct comparisons any explanation of the results obtained in our study can only be tentative. However there is suggestive evidence from earlier studies carried out in the marmoset that the effect of dietary cholesterol on tissue membrane fatty acid composition is too small to account for the effects seen in the current study. For example when we fed marmosets a reference type diet (P/M/S, 0.5:1:1), addition of 0.5% cholesterol resulted in only small inconsistent changes to both the n-3 and n-6 polyunsaturates of heart ventricle membranes (19). Furthermore in a separate study, marmosets fed a diet with a fatty acid composition identical to the atherogenic diet of the present study but with only 0.05% cholesterol were found to contain erythrocyte fatty acid patterns similar to the present study (16). For example the levels of fatty acids for the present study compared with the earlier study (16) were 18:2n-6 (13.0 ± 0.2, 12.6 ± 0.5) 20:4n-6 (11.4 ± 0.1, 11.9 ± 0.3) 22:5n-3 (4.1 ±

0.1, 3.7 ± 0.1) and 22:6n-3 (3.1 ± 0.1, 4.4 ± 0.2). Thus it is likely that cholesterol fed at a level of 0.2% (present study) will have caused only minimal changes in the erythrocyte membrane composition. It is interesting to note in this regard that the effect of cholesterol on plasma fatty acids may be species specific. For example 1% cholesterol added to the diet resulted in a decrease in plasma 20:4n-6 in rabbits (21). Interestingly, the dietary treatments employed in this study did not result in altered membrane cholesterol levels despite modulation of plasma levels (22).

The Lands equation (18 and Lands, W.E.M., personal communication) accurately predicted the increase in n-3 incorporation in the two ATH-based diets as well as the two REF-based diets (Fig. 1). Since deviations from predictions could be expected if factors other than those included in the equation (such as cholesterol) were influencing the equation, we take this to indicate that the increased incorporation of EPA into erythrocyte membranes in the two ATH-based diets was a result of reduced n-6 fatty acids in the diet rather than the presence of cholesterol. However it is clear that the equation did not accurately predict the level of n-6 HUFA in membrane total phospholipids of the ATH-based diets. This may indicate a perturbation in the membrane composition due to cholesterol.

Our interpretation of the results of this study is that in marmoset monkeys the incorporation of n-3 polyunsaturates into erythrocyte membranes is particularly sensitive to the presence of other fatty acids in the diet. Specifically we believe that the results indicate that the level of dietary 18:2n-6 over and above the level normally required to prevent essential fatty acid deficiency in animals (23) reduced n-3 incorporation. For example, in animals receiving EPA-supplemented diets the levels of 20:5n-3 in all erythrocyte phospholipid fractions from animals fed diets low in 18:2n-6 were about twice the level seen in membrane phospholipids of animals fed high 18:2n-6 diets. Interestingly the levels of 20:5n-3 in membranes of animals receiving unsupplemented atherogenic diets were 6–8 fold that seen in the reference or high 18:2n-6 group indicating that the effect applies to both endogenously synthesized, as well as supplemented, 20:5n-3. As has been mentioned, this effect can be predicted from the Lands equations (18 and Lands, W.E.M., personal communication) which quantify the interaction between n-3 and n-6 dietary fats.

Despite parallels that can be seen in some human studies, there have been few attempts to specifically test the strong effect of dietary 18:2n-6 on n-3 fatty acid incorporation into tissues or to measure these diet-induced changes in erythrocyte membranes in animals. However work with rats has shown that 20:5n-3 accumulates to a greater extent in serum and liver lipid fractions when n-3 fatty acids are fed in combination with saturated fatty acids than with 18:2n-6-rich diets (14,24). Indeed Lee and Sugano (25) have shown that in rats fed 18:3n-3, incorporation of 20:5n-3 into plasma and liver PC is more sensitive to dietary 18:2n-6 than is the incorporation of 22:6n-3.

The low 18:2n-6 (ATH) diet used in this study increased the proportion of 20:4n-6 found in erythrocyte total phospholipids and PE while it reduced the level of membrane 18:2n-6. We have observed similar effects in rats (26) implying that 18:2n-6 competes with both 20:4n-6 and 20:5n-3 for incorporation into the phospholipid molecule. The introduction of 20:5n-3 to both the ATH and REF diets further reduced the level of both 20:4n-6 and 18:2n-6 incorporation into the total phospholipids and PE fraction indicating a complex interrelationship between n-6 and n-3 fatty acids in these conditions. Indeed other studies have indicated that in rats, where a constant dietary intake of 18:2n-6 is maintained, dietary 18:3n-3 and 20:5n-3 can both independently result in increased incorporation of 18:2n-6 and decreased incorporation of 20:4n-6 into plasma and liver phospholipids (27). A study done in healthy women showed a similar relationship between these n-3 and n-6 polyunsaturated fatty acids associated with plasma cholesteryl esters (28).

A further effect observed in the 20:5n-3-treated groups was the reduction in the proportion of 20:3n-6, the precursor of the 1-series eicosanoids, to a level of 20–30% of that seen in the reference diet groups. Reductions in 20:3n-6 levels have been reported in tissue phospholipids of rats fed fish oil (29) and in the plasma of rheumatoid human subjects consuming 20:5n-3 (30); however, such changes were not observed in erythrocyte phospholipids (9). This may be a reflection of n-6 levels in the diets of these subjects, as it has been shown that it is possible to increase tissue 20:3n-6 levels in fish oil fed animals by enriching the diets with 18:3n-6 (31,32).

One of the most significant results for animals fed 20:5n-3 was the large increase in the proportion of 22:5n-3 in both the erythrocyte total phospholipids and in the PE fraction. However there was little change in 22:6n-3, Δ4 desaturase product of 22:5n-3. A similar trend has been observed in platelet phospholipids from atherosclerotic patients fed 20:5n-3 ethyl ester (33). Such an effect would be masked in subjects fed fish oils containing 22:6n-3. Whether this effect is due to poor conversion of 22:5n-3 or limited incorporation sites for 22:6n-3 into erythrocyte phospholipids is not clear from these results although the existence in rat liver of a Δ4 desaturase has recently been questioned (34). Certainly, the 30-wk feeding period employed in our study is sufficient to maximize diet-induced changes in erythrocyte membranes.

Overall, the most significant result of the present study was the increased incorporation of 20:5n-3 and 22:5n-3 in animals fed atherogenic diets compared to those consuming diets rich in 18:2n-6. Although directly comparable experiments have not been carried out in humans, there is evidence that incorporation of n-3 polyunsaturated fatty acids into plasma lipids and platelets of humans is inversely correlated with the level of dietary linoleate (35). Our results are therefore relevant to dietary studies on the effects of 20:5n-3 containing oils, on vascular disease risk factors (1,2) and arthritis (36).

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Monoenoic Fatty Acids in Human Brain Lipids: Isomer Identification and Distribution

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The carbon chain length distribution and the double bond positional isomer composition of the monoenoic fatty acids of the lipids of total human brain tissue have been determined using gas chromatography and gas chromatography/mass spectrometry of the fatty acid methyl and picolinyl esters. The even chain length monoenoic C_{16} to C_{28} fatty acids contain predominantly two positional isomer series, the n-7 and n-9 *cis* homologues, whose relative proportion varies significantly with chain length. The odd chain length long-chain fatty acids consist of n-8 and n-10 isomers, whereas the odd chain length very long-chain (more than 22 carbon) fatty acids are n-7 and n-9 isomers. ***Lipids 27,*** **177–180 (1992).**

The ratio of monoenoic fatty acids to saturated fatty acids in the lipids of human brain tissue is low at birth, rapidly increases when the process of myelination begins, and reaches a plateau by three years of age. Monoenoic fatty acids originate mostly from *de novo* synthesis and a small portion is derived from the diet. Two major double bond positional isomer series, the n-7 and n-9 homologues, have been identified. The reason for the existence of double bond positional isomers and whether each isomer has a different function is presently unclear. Studies with *cis* n-8 octadecenoic acid in animal (1) and human (2) tissues suggest that some positionally isomeric monoenoic fatty acids are metabolized by different routes. Monoenoic fatty acids may also play a role in the biosynthesis of saturated fatty acids (3).

Surprisingly, analyses of the double bond positional isomer series of the monoenoic fatty acids present in human brain, which would provide information to contribute to an understanding of the biosynthetic pathways of monoenoic fatty acids, have not been reported. A limited identification of isomer series of mixtures of monoenoic fatty acids has been achieved by GC analysis of the products after reductive ozonolysis. This approach, however, gives an average of all carbon chain lengths present.

A composite technique, described in this paper, coupled the identification capability of gas chromatography/mass spectrometry (GC/MS) and the quantitation capability of gas chromatography (GC) to achieve the dual requirements of analyzing double bond position and carbon chain length distribution of the total monoenoic fatty acids in whole human brain.

MATERIALS AND METHODS

Brain samples were obtained from male children aged 4, 5, 7 and 13 years who died in accidents, a male aged 53 years who suffered a cardiac tamponade, and a female aged 85 years who died from cardiorespiratory failure. The rat brain was obtained from a 14-day-old Porton rat, killed by decapitation.

Lipids were extracted according to Folch *et al.* (4) from 1-g samples of whole brain, containing similar amounts of white and grey matter, and were transesterified with 0.27 M sulfuric acid in methanol. Fatty acid methyl esters were isolated by preparative thin-layer chromatography (TLC) as previously described (5), and applied under nitrogen as 4 cm zones to silver nitrate impregnated silica gel 60 plates prepared by the method of Inomata *et al.* (6). A monoenoic fatty acid methyl ester standard was applied as reference at both edges of the plate and the chromatogram was developed in hexane/diethyl ether (95:5, v/v). After development, the plates were briefly air dried and sprayed with 0.2% (w/v) dichlorofluorescein in ethanol to locate the monoenoic methyl esters. That zone was scraped from the plate and eluted with 10 mL of chloroform/methanol (2:1, v/v). Dichlorofluorescein was removed by washing with dilute ammonia solution, and the chloroform solution was separated, dried and evaporated in a glass tube. This technique (argentation chromatography) separates monoenoic methyl esters from saturated (top of TLC plate) and polyenoic (bottom of TLC plate) methyl esters. To the monoenoic methyl ester fraction was added 1 mL of ethanol and 0.5 mL of 10% (w/v) potassium hydroxide in water, and the mixture was heated at 80°C for 4 hr. The solution was cooled, acidified with 0.5 mL concentrated hydrochloric acid, and extracted with 3 mL of hexane. The hexane extract, containing monoenoic fatty acids, was evaporated to dryness and 0.1 mL of thionyl chloride was added. After 5 min, excess thionyl chloride was removed in a stream of nitrogen and 0.05 mL of 3-pyridylcarbinol/acetonitrile (1:9, v/v) was added. The acetonitrile was removed in a stream of nitrogen after 10 min and 0.05 mL tetrahydrofuran was added. One μL of this solution was injected into the GC/MS.

The GC/MS was a JEOL JMS DX-303 single beam double focusing mass spectrometer (Tokyo, Japan) equipped with a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) and a JEOL JMA DA5000 data system. Esters were separated using a splitless injector, heated at 270°C, coupled to a Scientific Glass Engineering (SGE, Melbourne, Australia) BP-1 bonded phase silica column (12 m × 0.22 mm i.d., 0.25 μm phase thickness) directly inserted into the mass spectrometer source. For analyses of both methyl and picolinyl esters, the gas chromatograph was temperature programmed from 160°C to 320°C at 4°C/min, and then maintained at 320°C for 10 min. The mass spectrometer was operated in the electron impact mode at an ionization voltage of 70 eV and a source temperature of 250°C.

The GC was a Perkin Elmer Sigma 2 model (Norwalk, CT) equipped with a splitless injector system, a flame ionization detector and a Hewlett Packard 3396A integrator (Avondale, PA). Esters were separated on a nonpolar BP-1 bonded phase silica column (SGE, Melbourne,

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Abbreviations: amu, Atomic mass units; GC, gas-chromatography; GC/MS, gas chromatography/mass spectrometry; TLC, thin-layer chromatography.

Australia; 25 m × 0.33 mm i.d., 0.25 μm phase thickness) and a polar BPX70 (SGE) bonded phase silica column (25 m × 0.33 mm i.d., 0.25 μm phase thickness).

Samples of monoenoic fatty acids and methyl esters were obtained from NuChek Prep (Elysian, MN). Others, including 26:1n-9, 27:1n-9 and 28:1n-7 were synthesized (7).

RESULTS

Whole brain tissue, comprising approximately equal amounts of white and grey matter, from six normal individuals ranging in age from 4- to 85-years-old was extracted and the lipids were transesterified. The fatty acid methyl esters were resolved by TLC and analyzed by GC on both a polar and a non-polar capillary column, and then by GC/MS. The monoenoic fatty acid methyl esters were identified by GC/MS, by selective scanning of molecular ions and molecular ions less 32 amu (atomic mass units), and their percentage composition was determined by GC using an electronic integrator. The monoenoic fatty acids comprised 27.9% ± 0.9% S.D. of the total long-chain fatty acids (assuming a non-discriminatory response by the GC detector).

Each sample was then subjected to argentation chromatography and again analyzed by both GC and GC/MS. Both BP1 nonpolar and BPX70 polar chromatography columns were employed to ensure unambiguous assignment of the monoenoic fatty acid methyl esters. Small amounts of saturated fatty acids, principally methyl palmitate and methyl stearate, and other contaminants were present but they did not interfere with the analysis. The carbon chain length distribution of the monoenoic fatty acids is summarized in Table 1. To ensure that the relative composition of the monoenoic fatty acids was not significantly altered during argentation chromatography, the ratios of C_{26}/C_{18} monoenoic fatty acids before and after purification were compared. They were found to differ by between 4 and 10% in the 6 brain samples.

The double bond positional isomers of the mixture of monoenoic fatty acids were then identified by mass spectral fragmentation analysis of fatty acid derivatives. The 3-picolinyl esters first used by Harvey (8,9) and subsequently by Christie *et al.* (10–12) for the analysis of monoenoic long-chain fatty acids of animal and marine origin were chosen as the most suitable of the many available (13–17).

Initial attempts to prepare the picolinyl derivatives of the monoenoic fatty acids by reacting the acid chlorides with 3-pyridylcarbinol in acetonitrile were disappointing. The picolinyl derivatives, especially those of the very long-chain acids, were poorly soluble in acetonitrile. When this solvent was replaced by tetrahydrofuran, GC/MS response improved dramatically. The picolinyl derivatives of a series of monoenoic fatty acid standards (16:1n-7, 18:1n-9, 20:1n-9, 20:1n-12, 22:1n-9, 24:1n-9, 26:1n-9, 27:1n-9 and 28:1n-7) were prepared. The mass spectra of the picolinyl derivatives indicate cleavage of the terminal methyl group (loss of 15 amu) and then sequential cleavages of methylene groups (14 amu) until the double bond is reached, when there is a loss of 26 amu. In addition, the intensities of the two ions from cleavage two and three carbon atoms before the double bond are significantly higher. These ions have been rationalized by Harvey (8) and by Christie (18) as arising from cleavage after abstraction of an allylic hydrogen from either side of the double bond. This doublet of intense ions was shown to be the most reliable way of assigning the position of the double bond in the monoenoic fatty acid standards and then in the brain monoenoic fatty acids. For example, the spectrum of the picolinyl derivative of 19:1n-8, shown in Figure 1A, has a gap of 26 amu between *m/z* 262 and 288 and two strong ions at *m/z* 302 and 316, while the mass spectrum of the picolinyl derivative of 19:1n-10, shown in Figure 1B, has a gap of 26 amu between *m/z* 234 and 260 and two strong ions at *m/z* 274 and 288.

The GC/MS total ion chromatograms of the mixtures of picolinyl derivatives of the brain monoenoic fatty acids were extremely complex. Many peaks arose from side reactions of the reagents but, fortunately, few of them obscured the monoenoic fatty acid derivatives, which possessed intense ions at *m/z* 92, 108, 151 and 164, as well as prominent molecular ions. The picolinyl derivatives give broader peaks than the methyl esters and there is poorer separation of the isomers. Spectra taken on the leading and trailing edges of the peaks were, however, diagnostic of the two isomeric forms in all of the doublet peaks analyzed. Figure 1 shows two mass spectra, taken six seconds apart, of the picolinyl derivative of 19:1 fatty acid isolated from normal brain and assigned as the n-8 (A) and n-10 (B) isomers. The order of elution was the same as for methyl esters, *i.e.*, the lower numbered positional isomer eluted first on the non-polar column and last on the polar column. For this reason quantitation of the isomeric forms of the monoenoic fatty acids was performed using the methyl esters. Figure 2 shows the purified monoenoic fatty acid methyl esters from a normal adult brain and their isomer assignments. The monoenoic fatty acid isomer distribution of the six brain samples are presented in Table 2. A realistic level of isomer detection would be 5% of the total for each carbon chain number, so minor amounts of other isomers may well be present.

The monoenoic fatty acid composition of a 14-day-old rat brain (near the onset of myelination) was determined by the same procedure (data not shown), and the only noticeable difference from normal human brain was a

TABLE 1

The Distribution of Monoenoic Fatty Acids in Brain[a]

Carbon number	Composition (%)	
	Mean	S.D.
16	3.53	0.40
17	0.65	0.13
18	81.87	2.69
19	0.34	0.04
20	4.42	0.61
21	0.14	0.05
22	0.47	0.08
23	0.20	0.12
24	5.20	1.31
25	1.20	0.46
26	1.45	0.43
27	0.52	0.16
28	0.14	0.08

[a]The percentage distribution of monoenoic fatty acid isomers with the same carbon chain length to the total monoenoic fatty acids found in six specimens of human brain.

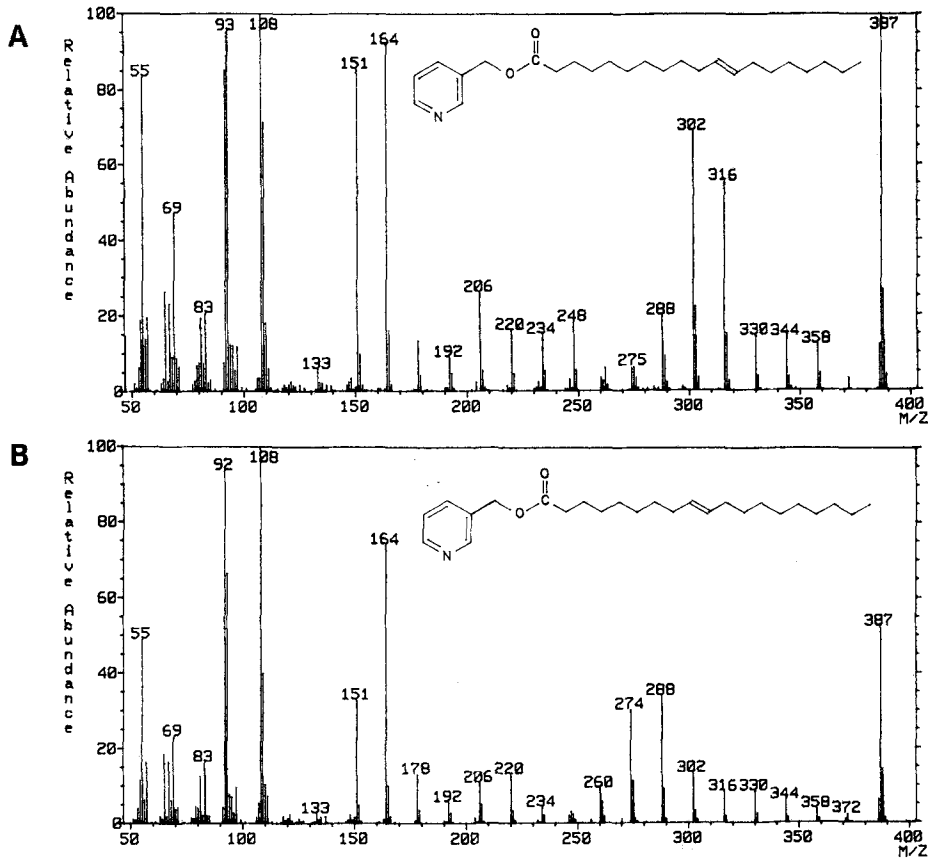

FIG. 1. Mass spectra of picolinyl derivatives of isomers of 19:1 fatty acid. The electron impact (70 eV) mass spectra shown are those of the picolinyl derivatives of (A) 19:1n-8 fatty acid (top) and (B) 19:1n-10 fatty acid (bottom).

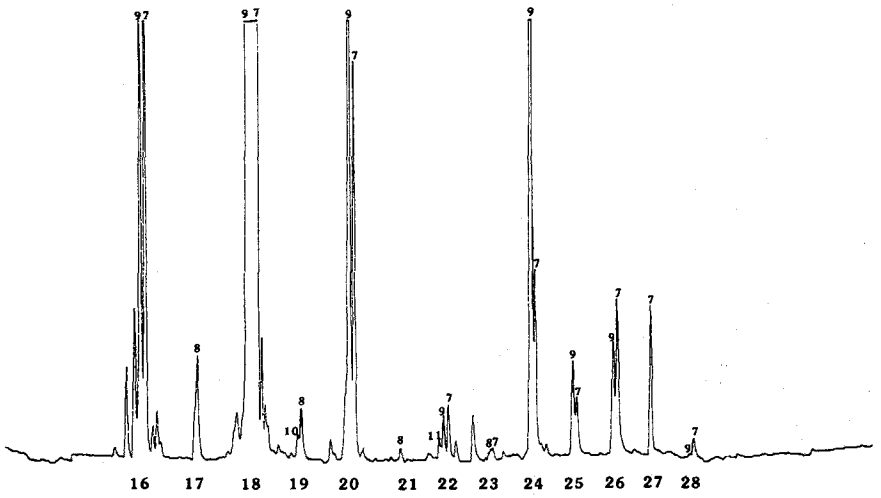

FIG. 2. Gas chromatography of the methyl esters of monoenoic fatty acids of human brain. The recorder tracing is of the purified monoenoic fatty acid methyl esters obtained from an adult human brain. The peaks are labelled with the isomeric series on top and the carbon chain number below.

TABLE 2

Isomer Composition of Monoenoic Fatty Acids in Human Brain[a]

Carbon number	Isomer (n-x)	Composition (%)	
		Mean	S.D.
16	7	51.5	2.5
	9	48.5	2.5
17	8	100	
18	7	11.4	2.1
	9	88.6	2.1
19	8	66.5	1.5
	10	33.5	1.5
20	7	27.2	1.9
	9	72.8	1.9
21	8	100	
22	7	40.4	5.9
	9	39.6	3.6
	11	20.0	4.1
23	7	47.8	4.2
	8	52.2	4.2
24	7	13.1	1.7
	9	86.9	1.7
25	7	30.5	5.5
	9	69.5	5.5
26	7	52.7	5.0
	9	47.3	5.0
27	7	100	
28	7	77.7	4.5
	9	22.3	4.5

[a]The distribution (expressed as a percentage) of the monoenoic fatty acid isomers with the same carbon chain length in six specimens of human brain.

greatly reduced proportion of 16:1n-7 fatty acid, which probably reflects the diet different from that of humans.

DISCUSSION

The fatty acid composition and isomer distribution of human brain that we measured at ages 4, 5, 7, 13, 53 and 85 years were remarkably consistent. This does not contradict the accepted view that there is a reduction in monoenoic fatty acids with age because percentage composition, and not fatty acid concentration, per g of brain tissue was determined. The even numbered carbon chain monoenoic fatty acids contain predominantly n-7 and n-9 series isomers, which may originate from dietary palmitoleic acid (16:1n-7) and oleic acid (18:1n-9). The ratio of amounts of the two isomer series at each even carbon chain number is not constant, but varies in a cyclical pattern with increasing carbon chain number. The n-7/n-9 ratio has maxima at 16, 22 and 28 carbon atoms and minima at 18 and 24 carbon atoms, which indicates that the two series are metabolized differently.

The odd numbered carbon chain monoenoic long-chain fatty acids contain mostly n-8 and n-10 isomers. Long-chain n-10 fatty acids are biosynthesized in skin sebaceous glands and in cultured fetal rabbit lung tissue (19). The origins of the n-8 and n-10 isomers in the brain are therefore probably biosynthetic and the fatty acids are not directly derived from the diet. The odd numbered carbon chain monoenoic very long-chain fatty acids (*e.g.*, 25:1 and 27:1) consist only of n-7 and n-9 isomers. These result from one carbon shortening by α-oxidation of the longer, even chain, monoenoic fatty acids.

No *trans* monoenoic fatty acids were identified for which there is an explanation. Studies by Ohlrogge *et al.* (2) had shown that the most abundant *trans* octadecenoic fatty acid in brain, the n-8 isomer, was less than 1% of the total octadecenoic fatty acids, *i.e.*, well below our detection limit. Furthermore, Cook (20) had shown that no *trans* monoenoic fatty acids longer than C_{18} were present in brain.

Brain is a complex mixture of organelles, cells and cellular components including neurons, synaptosomes, astrocytes, oligodendrocytes, myelin, microsomes and mitochondria. The method described has been successful in defining the monoenoic fatty acid composition and distribution of a representative sample of these. Processes involving synthesis and differential metabolism of monoenoic fatty acids, either in normal brain or diseased brain, may occur in specific cell (or cell component) types. Provided that methods will become available to isolate purified cells (or cell components), our method can be scaled down as much as ten-fold to adjust for smaller sample sizes.

ACKNOWLEDGMENTS

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Effects of Polyphenolic Natural Products on the Lipid Profiles of Rats Fed High Fat Diets

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Male Wistar rats were fed a high fat diet (HFD) containing 2.5% cholesterol and 16% lard supplemented with polyphenolic natural products namely quercetin, morin or tannic acid (100 mg/rat/day) for 4, 7 and 10 wk. Rats fed HFD without the supplements served as control. The effects of these compounds on blood lipid profiles, enzymes, liver fat and aorta of the rat were studied. In rats fed HFD containing tannic acid, plasma total cholesterol (TC), low density lipoprotein cholesterol (LDLC) and triglyceride (TG) were reduced by 33.3%, 29.6% and 65.1%, respectively, at week 10. High density lipoprotein cholesterol (HDLC) concentration was not altered. Fat deposition was also decreased in the liver of these rats. Morin significantly reduced plasma TG (65.1%) and liver fat only at week 7 while at week 10 it reduced plasma TC and LDLC by 30.9% and 29.3% respectively. The plasma HDLC concentration was increased by 47.3% at week 4 but no effect was seen at weeks 7 and 10. In the rats fed HFD containing quercetin, plasma HDLC was increased by 28.6% at week 7 but at week 10, plasma LDLC was increased by 21.2%. Quercetin did not cause any significant changes on the plasma TC, TG and liver fat at weeks 4, 7 and 10. Plasma alanine aminotransferase, alkaline phosphatase and bilirubin in control and treated groups were not significantly different. However, hepatic lipase activity in rats fed tannic acid was significantly lower. Aortae of all groups of rats showed no abnormalities. The present report indicates that tannic acid and morin are effective in reducing plasma and liver lipids when supplemented with a high fat diet in rats.

Lipids 27, 181-186 (1992).

Coronary heart disease (CHD) is one of the most prevalent causes of death in the United States (1). Hypercholesterolemia is an important etiological factor in CHD. Studies have shown that the risk of developing CHD is linearly related to serum cholesterol concentration (2) and low density lipoprotein cholesterol (LDLC; ref. 3), while high density lipoprotein cholesterol (HDLC) exerts a protective effect (4). Other complications of hypercholesterolemia include the development of premature atherosclerosis and atherothrombotic brain infarction (1). Dietary lipids have been reported to cause atherosclerosis in various animal models (5-8). In rats, cholesterol feeding increased low density lipoprotein (LDL) and decreased high density lipoprotein (HDL; ref. 9).

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Abbreviations: ALAT, alanine aminotransferase; ANOVA, analysis of variance; AP, alkaline phosphatase; ATP, adenosine triphosphate; BF_3, borontrifluoride; BHT, butylated hydroxytoluene; CHD, coronary heart disease; EDTA, ethylenediaminetetraacetate; G1, group 1; G2, group 2; G3, group 3; G4, group 4; G5, group 5; HDLC, high density lipoprotein cholesterol; HFD, high fat diet; HFD + M, high fat diet + morin; HFD + T, high fat diet + tannic acid; HFD + Q, high fat diet + quercetin; LDLC, low density lipoprotein cholesterol; NADH, reduced nicotinamide adenine dinucleotide; ND, normal diet; TC, total cholesterol; TG, triglyceride.

The polyphenolic natural products namely flavonoids and tannic acid (hydrolyzable tannin; Fig. 1) are ubiquitous in plants and are easily accessible to animals and man through their diets (10). It is estimated that the average American (USA) adult dietary intake of these natural products is about 1 g/day comprised of mixed flavonoids (11). These polyphenolic substances have been effectively used to treat certain human diseases (12,13). Flavonoids increased the survival time of rats when fed together with either a thrombogenic or atherogenic diet (14). In *in vitro* studies, they have exhibited antithrombotic effects (15).

Quercetin, found commonly in vascular plants (16), reduced plasma total cholesterol (TC) when maintained in colloidal suspension *in vitro* (17) but not plasma total cholesterol (TC) in rats (18). It is also non-toxic (19) and non-carcinogenic (20). Morin is structurally related to quercetin (a flavonol; Fig. 1). Tannins are commonly found in food (21) and in herbal medicine (22). Tannic acid (a hydrolyzable tannin) occurs in the bark and fruits of many plants (23) and is non-toxic when fed to rats at dietary levels of less than 5% (24).

In view of the limited reports on the biological effects of polyphenolic natural products on rats fed atherogenic diets (14), it was felt useful to carry out a study in order to evaluate the effects of some of these compounds namely quercetin, morin and tannic acid when supplied as food supplements (100 mg/rat/day) to rats fed high cholesterol and lard diets. The single dose was selected because our preliminary investigation (data not shown) had indicated it to be the most ideal dosage to use. Single dosage studies

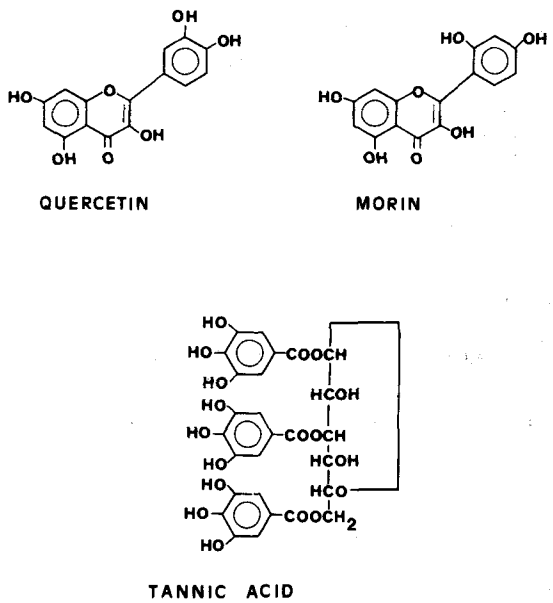

FIG. 1. Structure of polyphenolic compounds used in the present study.

have been used to study the effects of other compounds on serum and liver cholesterol (25,26). Any beneficial effects of these natural products may provide a basis for future investigations for the possible treatment of lipid disorder resulting from high fat dietary intake. Their reported non-toxicity in animals rendered the compounds useful for the present study.

MATERIALS AND METHODS

Chemicals. Cholesterol was purchased from E. Merck (Darmstadt, Germany). Morin and tannic acid were obtained from Extrasarsyntex (Ganey, France). Quercetin was supplied by Sigma Chemical Co. (St. Louis, MO).

Animals and treatment. Male Wistar rats, body weight approximately 180 g were used. The rats were divided into five groups, each group comprising nine rats as follows: Group 1 (G1)—normal diet; Group 2 (G2)—high fat diet (HFD) without supplement; Group 3 (G3)—HFD with quercetin; Group 4 (G4)—HFD with morin; and Group 5 (G5)—HFD with tannic acid. The compositions of the diets and their fatty acid contents are given in Tables 1 and 2, respectively. The normal diet was adopted from Griffith's (27) earlier report because it was necessary to use a natural diet which contained a low level of flavonoids and other phenolic compounds. During the first week of the experiment, each rat consumed about 7 g/day of the powdered diet. This amount was increased by 1 g weekly. Each polyphenol (100 mg/rat/day) was mixed thoroughly into the diet prior to feeding. At the end of 4, 7 and 10 wk, 3 rats were taken from each group, weighed and then decapitated. Whole blood was collected into tubes containing ethlenediaminetetraacetate (EDTA) and centrifuged to obtain plasma. The plasma was either analyzed immediately or stored at −70°C until analyses were carried out.

Tissue preparation. The fresh liver from each rat was partly cut into 2-mm sections and preserved in 10% formalin. Further 10-μm sections were made and stained with Oil Red 'O' for examination of fat distribution. A sample of liver (1 g) was used for the determination of TC and triglyceride (TG). TC and TG were extracted according to the method of Haug and Hostmark (28). Hepatic lipase was extracted from the remaining fresh liver according to our earlier reported method (29). Thoracic aorta was removed from each rat and preserved in 10% formalin. Each aorta was subsequently cut into 10 equal sized sections, dehydrated and paraffin embedded. Using a microtome, each section was further cut into 8-μm slices. The slices were stained with hemotoxylin and eosin (H and E) and examined under a light microscope for evidence of atherosclerotic changes.

Lipid analysis. The concentrations of plasma LDLC, HDLC, TC, and TG and of liver TG and TC were determined using commercially available diagnostic kits from Boehringer Mannheim GmbH (Singapore) according to the protocol supplied.

Briefly, cholesteryl esters were enzymatically cleaved by cholesterol esterase to liberate cholesterol and free fatty acids. The enzyme-liberated cholesterol and the endogenous free cholesterol were oxidized by cholesterol oxidase to form Δ^4 cholestenone and hydrogen peroxide (H_2O_2). The H_2O_2 generated was reacted with 4-aminophenazone and phenol (catalyzed by peroxidase) to form a pink complex. The color intensity was read at 500 nm.

LDL in plasma was reacted with polyvinyl sulfate which resulted in the formation of the LDLC precipitate. The supernatant was taken to determine the total cholesterol concentration. From the difference between plasma TC and TC in the supernatant after centrifugation, the LDLC value was calculated.

For the determination of HDLC, phosphotungstic acid and $MgCl_2$ were added to the sample to precipitate chylomicrons, very low density lipoprotein (VLDL) and LDL. The HDL remained in the supernatant after centrifugation and the cholesterol content was determined as described above.

TG was hydrolyzed by lipase to form free fatty acids and glycerol. The glycerol was reacted with glycerol kinase in the presence of adenosine triphosphate (ATP) to form glycerol-3-phosphate. The glycerol-3-phosphate was oxidized by glycerol phosphate oxidase to form dihydroxyacetonephosphate and H_2O_2. The H_2O_2 was complexed with 4-aminophenazone and phenol, and the pink color formed was read at 500 nm.

Enzyme and bilirubin analysis. Activities of the enzymes alkaline phosphatase (AP) and alanine aminotransferase (ALAT) in plasma, hepatic lipase, and plasma total bilirubin concentrations were determined using diagnostic kits from Boehringer Mannheim GmbH.

Briefly, in the determination of AP, the substrate *p*-nitrophenyl phosphate was hydrolyzed to phosphate and *p*-nitrophenol by the enzyme. The formation of *p*-nitrophenol was then measured at 405 nm. In the determination of ALAT, the sample was added to the test reagent which contained a mixture of α-oxoglutarate and L-alanine as substrate. α-Oxoglutarate and L-alanine were acted upon by ALAT to form glutamate and pyruvate,

TABLE 1

Compositions of the Diets

Contents	Normal diet (g/100 g)	High fat diet (g/100 g)
Wheat flour	70.7	52.6
Milk powder	23.6	23.2
Dried yeast powder	3.5	3.5
Sodium chloride	1.2	1.2
Multivitamins[a]	1.0	1.0
Cholesterol powder	0	2.5
Pork lard	0	16.0

[a]Vitamin composition is given in our earlier report (29).

TABLE 2

Fatty Acid Composition of Experimental Diets and Lard (% of total fatty acids)

Fatty acid	Normal diet	High fat diet	Lard
10:0	4.0	2.0	—
12:0	4.8	2.2	—
14:0	12.3	6.3	2.1
16:0	27.3	27.9	28.3
18:0	10.3	12.6	14.9
18:1n-9	18.2	29.6	37.4
18:2n-6	9.2	9.7	10.6
18:3n-6	5.7	3.5	—

respectively. The pyruvate was further converted to lactate in an irreversible reaction by lactate dehydrogenase in the presence of reduced nicotinamide adenine dinucleotide (NADH) and the decrease in absorbance due to the utilization of NADH was followed at 340 nm.

Hepatic lipase hydrolyzed triolein (in test reagent) to form monoglyceride and oleic acid. The decrease in turbidity due to the hydrolysis of triolein was also measured at 340 nm.

Total bilirubin in plasma was coupled with diazotized sulfanilic acid in the presence of caffeine to give an azo dye which absorbed at 580 nm.

Diet fatty acid analysis. The lipids in the rat diets were extracted using chloroform/methanol (2:1, v/v) containing 0.02% butylated hydroxytoluene (BHT) as an antioxidant. The chloroform layer, which contained the lipids, was removed and saponified with methanolic KOH and concentrated HCl. The free fatty acids were extracted into n-hexane and then methylated using borontrifluoride (BF_3)/methanol (30). The fatty acid methyl esters were analyzed on a Varian model 3400 gas chromatograph equipped with a flame ionization detector and a fused silica capillary column (DB-1; from J & W Scientific, Folsom, CA). A programmed run over the temperature range of 150°-250°C, on-column injection, and oxygen-free nitrogen as carrier gas were used.

Statistical analysis. Values of the biochemical parameters in each group are expressed as mean ±SEM. One way analysis of variance (ANOVA; ref. 31) followed by Duncan's Multiple-Range Test (32) were used to evaluate the significance of differences found between mean values. P values < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Food intake in all the five groups of rats were unaffected by the experimental diet. The rats in each group consumed all the allotted amount of food daily. Since hypercholesterolemia was induced in the experimental group of rats by feeding cholesterol and lard, the G1 normal diet (ND) rats were used as a comparison to G2 (HFD) in order to determine at which time interval (week 4, 7 or 10) blood cholesterol was significantly increased. Feeding of the HFD for 4 wk did not significantly induce hypercholesterolemia in G2 (Fig. 2). However, at week 7 the plasma TC in G2 was significantly increased in comparison to G1. The plasma cholesterol concentration was presumably stabilized in G2 after 7 wk because there was no further increase at week 10. In contrast to this, plasma TC in G1 did not fluctuate significantly from week 4 to 10 with an average concentration of 76.4 ± 2.1 mg/100 mL.

The G2 (HFD without supplement) rats were used as control for the test groups G3–G5. In G5, plasma TC was significantly reduced by 26.4% and 33.3% at week 7 and 10, respectively (Fig. 2). In G4, plasma TC was only significantly reduced at week 10 (30.9%), thus indicating that morin is able to reduce plasma cholesterol upon prolonged intake.

All the rats showed progressive increase in body weight from weeks 4–10. However, body weights did not differ significantly amongst groups (G1–G5) during the 10 wk period (Table 3). Therefore, the reduction in plasma TC in G4 and G5 cannot be attributed to weight loss or lower

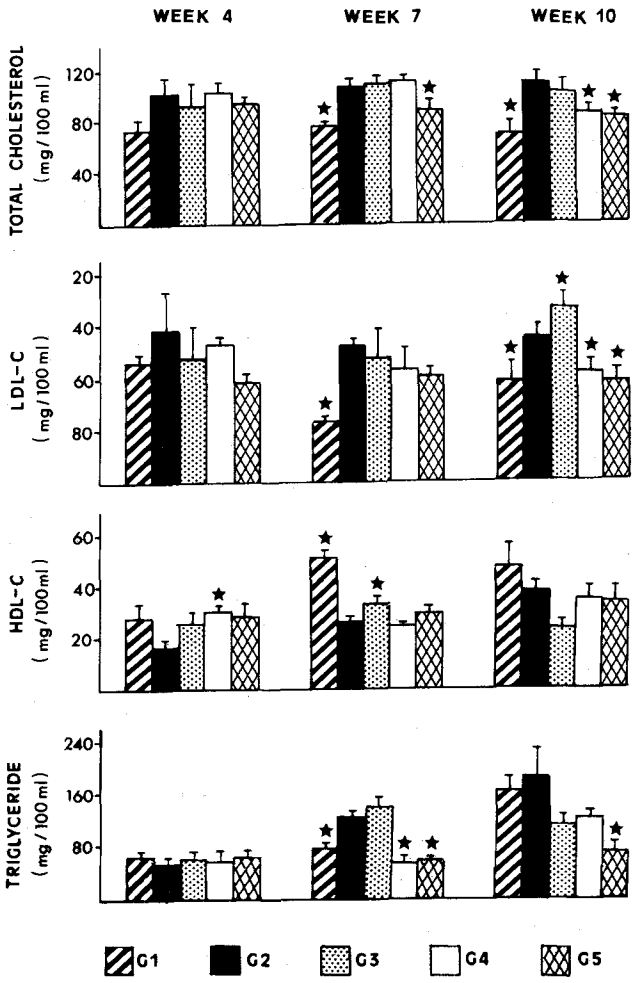

FIG. 2. Concentration of plasma total cholesterol, LDL-C, HDLC-C and triglyceride. Values are expressed as mg/100 mL plasma. ★ denotes a statistically significant difference between G2 and other groups ($p < 0.05$).

TABLE 3

Average Body Weight Increases (measured as percentage)[a]

Animal groups	% Increase in body weight		
	Week 4	Week 7	Week 10
G1 (ND)	36.3 ± 1.6[a]	58.6 ± 14.1[a,b]	89.2 ± 9.5[a]
G2 (HFD)	48.3 ± 5.3[a]	73.0 ± 5.4[a]	85.6 ± 7.8[a]
G3 (HFD + Q)	38.4 ± 6.3[a]	74.8 ± 1.8[a]	100.0 ± 3.1[a]
G4 (HFD + M)	47.6 ± 11.0[a]	61.7 ± 4.6[a]	100.0 ± 8.2[a]
G5 (HFD + T)	46.5 ± 5.6[a]	73.0 ± 8.2[a]	88.5 ± 5.7[a]

[a]In each experiment, values in the same column without common superscript letters denote significant difference ($p < 0.05$). Values are mean ± SEM of three animals.

weight gain of the rats in these two groups. In contrast, large reductions in body weight have been associated with reduced blood lipids (33). Quercetin (G3) did not cause any significant changes on plasma TC concentration at weeks 4, 7 or 10 (Fig. 2). Basarkar and Hatwalne (18) have also reported that feeding of quercetin did not lower plasma

TC in rats. However, in *in vitro* experiments, quercetin reduced plasma cholesterol when maintained in colloidal suspension (17).

The plasma LDLC concentration in G1 was significantly lower than in G2 at weeks 7 and 10 (Fig. 2). At week 10, LDLC in G4 and G5 were significantly reduced by 29.3% and 29.6%, respectively while in G3 a significant increase occurred (21.2%; Fig. 2). No significant differences were observed at weeks 4 and 7. Plasma concentration of LDLC is largely dependent on the rates of its production and removal from the circulation (34). LDLC is produced from very low density lipoprotein (VLDL) which is secreted by the liver and most of the plasma LDLC is removed *via* a hepatic receptor mediated process (35,36). A reduction in plasma LDLC by morin or tannic acid as observed in the present study suggests that these compounds may interfere with either one of these processes or both.

Plasma HDLC concentration in G1 and G3 were significantly higher (50.0% and 28.6%, respectively) than in G2 at week 7, but not at weeks 4 or 10 (Fig. 2). In G4 though HDLC was significantly increased by 47.3% at week 4, this effect was not sustained in the subsequent weeks (7 and 10). No significant difference was observed in plasma HDLC concentration in G5 when compared to control (G2) at weeks 4, 7 or 10. Mahley and Holcombe (9) have reported an increase in LDL and a decrease in HDL following cholesterol feeding in the rats. Cholesterol transport to extrahepatic tissues is primarily ensured by LDL while HDL has an important role in reversing the cholesterol transport process, whereby excess cholesterol is removed from peripheral tissues to the liver for excretion (37).

It is interesting to note that tannic acid is able to reduce LDLC and TC without affecting the HDLC level. Thus, there is a significant increase in the HDLC to TC ratio for this group of rats when compared to the control group (G2; Table 4). This ratio is suggested to be a more useful index for determining the quality and effects of fat on health. We have also recently reported a high ratio value on rats fed refined, bleached and deodorized palm oil (29).

Joslyn and Glick (24) have shown that tannic acid is non-toxic at dietary concentration of 5% or less. They also observed that tannic acid toxicity depended on the initial body weight of the rats used. Rats with a higher initial body weight showed greater resistance to tannic acid toxicity. In the present investigation we have fed less than 1.5% dietary concentration of tannic acid to rats with an initial body weight of about 180 g. We have observed no weight loss nor death throughout the experimental period and all the rats appeared to be normal and healthy.

No significant differences were observed in plasma TG concentration between all groups at week 4. At week 7, plasma TG concentration in G2 was significantly higher than in G1 (52.4%) indicating that the increase of TG in G2 is of dietary origin and the TG level appeared to be stabilized from week 7 onward because no significant difference was observed at week 10. The plasma TG of G4 and G5 were lower than G2 by 65.1% and 56.6%, respectively, at week 7. At week 10, plasma TG in G5 was further reduced (65.1%). It has been reported that enrichment of diet with n-3 fatty acids also reduces plasma TG concentrations in the rat (38). Perhaps morin or tannic acid may have acted in the similar process as the n-3 fatty acids.

The significant increase in liver weight (Table 5) without a significant increase in body weight (Table 3) of rats in G2–G5 in comparison to G1 (normal diet fed rats) is probably due to higher fat deposition resulting from a higher fat intake. Microscopic examination of the liver supports this conclusion for there was a minimal amount of fat deposited in the liver of normal diet fed rats (Fig. 3a). The tannic acid fed group (G5) showed less fat deposition (but higher than G1) in the liver when compared to G2–4 (Fig. 3b & c). This indicates that tannic acid is able to reduce the infiltration of fat into the liver cells of rats fed high fat diet.

Analysis of the liver TG and TC content at week 7 (Table 6) showed liver TC in G1 and G5 to be significantly lower than G2. In G1 and G4 the liver TG content was significantly lower than in G2 (Table 6). Although there was a significant decrease in liver TG of G4, this effect was not manifested in liver weight or liver fat distribution. Data for liver cholesterol and TG contents at weeks 4 and 10 were not obtained. Sugano *et al.* (39) observed reductions in liver cholesterol concentration of rats when fed with chitosan and cholesterol, and they have explained the reduction as a result of chitosan interfering with cholesterol absorption. It may be pertinent to suggest that tannic acid may also interfere with cholesterol absorption.

The liver plays an important role in the catabolism of cholesterol. Conversion of cholesterol to bile acids occurs exclusively in the liver and represents the major pathway for the elimination of cholesterol from the body (40). Retention of biliary constituents due to bile duct obstruction was reported to show an initial rise in plasma bilirubin accompanied by an increase in AP and ALAT (41) as

TABLE 4

Ratio of HDL Cholesterol to Total Cholesterol[a]

Animal groups	Week 4	Week 7	Week 10
G1 (ND)	0.35 ± 0.06[a]	0.67 ± 0.12[b]	0.68 ± 0.14[b]
G2 (HFD)	0.28 ± 0.04[a]	0.24 ± 0.05[a]	0.34 ± 0.09[a]
G3 (HFD + Q)	0.29 ± 0.04[a]	0.30 ± 0.05[a]	0.23 ± 0.04[a]
G4 (HFD + M)	0.27 ± 0.03[a]	0.22 ± 0.02[a]	0.25 ± 0.05[a]
G5 (HFD + T)	0.30 ± 0.05[a]	0.35 ± 0.06[b]	0.45 ± 0.06[b]

[a]In each experiment, values in the same column without common superscript letters denote significant difference ($p < 0.05$). Values are mean ± SEM of three animals.

TABLE 5

Liver Weight (g/100 body weight)[a]

Animal group	Week 4	Week 7	Week 10
G1 (ND)	2.9 ± 0.1[b]	2.9 ± 0.1[a]	2.6 ± 0.1[b]
G2 (HFD)	3.5 ± 0.1[a]	3.3 ± 0.2[a]	3.3 ± 0.2[a]
G3 (HFD + Q)	3.5 ± 0.2[a]	3.3 ± 0.1[a]	3.2 ± 0.1[a]
G4 (HFD + M)	3.3 ± 0.3[a,b]	3.3 ± 0.3[a]	3.3 ± 0.1[a]
G5 (HFD + T)	3.3 ± 0.1[a,b]	2.9 ± 0.1[a]	3.3 ± 0.1[a]

[a]In each experiment, values in the same column without common superscript letters denote significant difference ($p < 0.05$). Values are mean ± SEM of three animals.

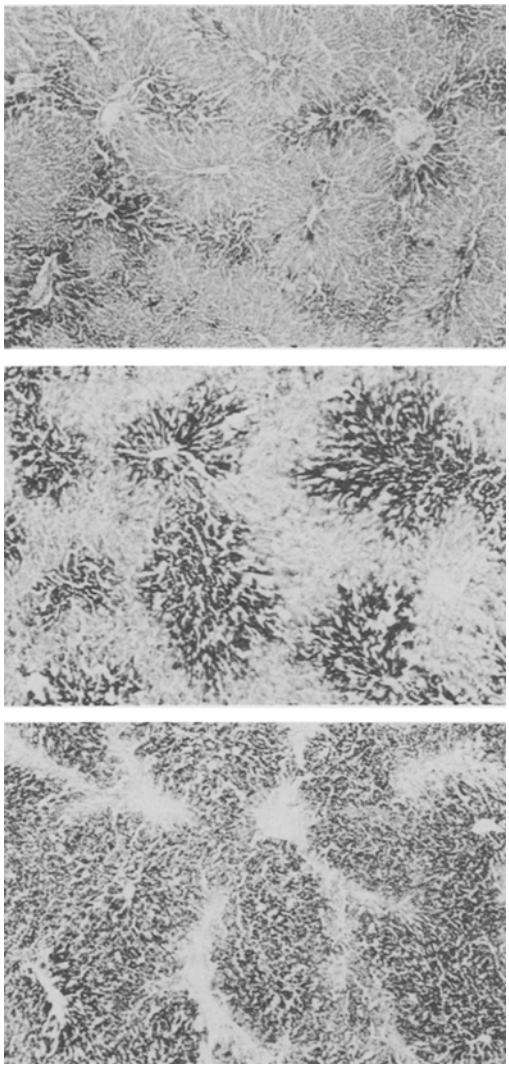

FIG. 3. a) Liver of a rat fed normal diet for 10 wk shows minimal deposition of fat in the hepatocytes. (Oil Red O stain, 75×). b) Liver of a rat fed high fat diet supplemented with tannic acid (100 mg/rat/day) for 10 wk shows moderate deposition of fat in the hepatocytes with sparing of the centrilobular hepatocytes. (Oil Red O stain, 80×). c) Liver of a rat fed high fat diet without polyphenolic compounds supplement for 10 wk shows severe and panlobular involvement of hepatocytes. (Oil Red O stain, 75×). Liver of rats fed high fat diet with quercetin (100 mg/rat/day) and morin (100 mg/rat/day) for 10 wk also showed similar appearance.

TABLE 6

Liver Total Cholesterol and Triglyceride Concentration at Week 7[a]

Animal group	Total cholesterol (mg/g liver)	Triglyceride (mg/g liver)
G1 (ND)	2.2 ± 0.1[b]	15.9 ± 1.5[b]
G2 (HFD)	26.6 ± 0.9[a]	42.9 ± 10.9[a]
G3 (HFD + Q)	18.6 ± 2.6[a]	45.1 ± 3.6[a]
G4 (HFD + M)	22.3 ± 2.2[a]	29.5 ± 2.0[b]
G5 (HFD + T)	15.9 ± 0.9[b]	33.1 ± 5.9[a]

[a]In each experiment, values in the same column without common superscript letters denote significant difference ($p < 0.05$). Values are mean ± SEM of three animals.

well as cirrhosis of the liver (42). Others have shown increases in tissue levels of AP due to diet induced hyperlipidemia (43). In contrast, the present study showed no significant differences in the activities of plasma AP, ALAT and bilirubin concentration in all groups of rats. The activities ranged from 47.2 ± 5.2 to 52.8 ± 3.2 U/L and 40.3 ± 4.8 to 45.1 ± 4.1 U/L for ALAT and AP, respectively. The total bilirubin concentration ranged from 11.0 ± 2.5 to 15.2 ± 2.7 mg/100 mL. Thus, these results indicate that feeding of a diet high in cholesterol and lard content or a cholesterol and lard diet supplemented with polyphenolic compounds does not cause bile retention or liver cirrhosis.

Hepatic lipase activity in G1 and G5 were significantly lower than in G2 at weeks 7 and 10 (Fig. 4). Jansen and Hulsmann (44) have suggested an involvement of hepatic lipase in the delivery of cholesterol to the liver. They have proposed that cholesterol is taken up from peripheral cells by a phospholipid rich HDL and subsequently delivered to the liver *via* a mechanism involving the depletion of phospholipid from HDL by hepatic lipase. Along this line, others have proposed that the increase in hepatic lipase activity could contribute to increases in liver cholesterol (45). Our findings are in agreement with these reports as increased hepatic lipase activity in G2, G3 and G4 correlates well with increased liver cholesterol concentration at week 7 (correlation coefficient, $r = +0.632$).

Atherosclerotic plaques were not observed in the aorta of the five groups of rats. Microscopic examination of the H and E stained paraffin sections of the thoracic aorta showed smooth, intact intimal lining with no evidence of raised fatty plaques or proliferation of smooth muscle cells, thereby demonstrating the absence of atherosclerosis in all the groups of experimental rats.

In summary, the present study has shown that both tannic acid and morin can cause favorable changes in plasma lipid profiles of the type that have been correlated with CHD. Tannic acid reduced fat deposits in liver cells at nontoxic concentrations. Thus, tannic acid has a potential to avert complications arising from high fat intake. Further studies need to be carried out to unravel the mechanism of the observed hypolipidemic effect of tannic acid.

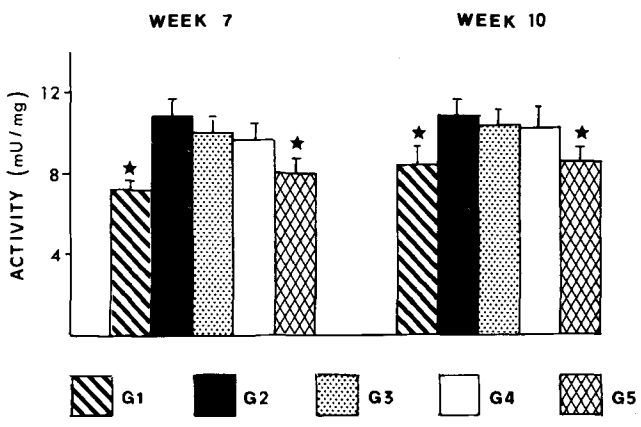

FIG. 4. Hepatic lipase activity of rats at weeks 7 and 10. Values are expressed as mU/mg protein. ★ denotes a statistically significant difference between G2 and other groups ($p < 0.05$).

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Subterminal Hydroxylation of Lauric Acid by Microsomes from a Marine Fish

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Microsomes from the liver of sea bass (*Dicentrarchus labrax*) were shown to hydroxylate lauric acid at subterminal positions. The cytochrome P-450 system converted lauric acid to several mono-hydroxylated metabolites including ω-1 hydroxylaurate, which was the major metabolite (44% of total products). In addition, ω-2, ω-3, ω-4 and a small amount (2.3%) of ω hydroxylaurates were found. Reaction products were identified using thin-layer chromatography (TLC) and gas chromatography/mass spectrometry (GC/MS). Oxidation reactions were dependent upon O_2 and NADPH, and did not occur with boiled microsomes or in the presence of a mixture of CO/O_2. Hydroxylation proceeded linearly up to 20 min at 28°C for protein concentrations below 380 μg. Treatment of fish with benzo(*a*)pyrene (BP) (20 mg/kg) drastically increased xenobiotic metabolism (ECOD, EROD and BPMO activities), but no difference in laurate hydroxylase activity was observed between untreated and treated fish. Starvation strongly enhanced laurate hydroxylase activity, and resumption of feeding reduced by half this increase of activity. In all of the experiments we did not observe any modification of the regioselectivity of lauric acid hydroxylation by this microsomal in-chain hydroxylating system. We suggest that cytochrome P-450 enzymes involved in lauric acid and xenobiotics metabolism are regulated independently.

Lipids 27, 187-191 (1992).

Cytochrome P-450 enzymes are involved in many essential reactions, including the metabolism of fatty acids. The physiological significance of the metabolism of fatty acids, prostaglandins and leukotrienes by monooxygenases remains uncertain. Lauric acid hydroxylases are widely distributed and cytochrome P-450 is a component of these monooxygenase systems found in most living organisms. Differences, depending on enzyme source and isozyme multiplicity, appear in the regiospecificity of the oxidation of lauric acid. Hydroxylation occurs at the terminal methyl end and/or in the chain of the molecule. A more pronounced difference exists between organisms which catalyze ω hydroxylation of lauric acid, *i.e.* mammals (1), amphibians (2), plants (Leguminosae) (3), insects (4), and yeasts (5), and those which hydroxylate fatty acids exclusively in the aliphatic carbon chain, *i.e.* fish (6), plants (other than Leguminosae) (7), fungi (8), and bacteria (9).

The specificities and physiological roles of these enzyme systems are not completely understood. It has been reported that rat liver microsomes probably contain three cytochrome P-450 systems involved in ω and ω-1 hydroxylation of fatty acids (10). Rabbit liver microsomes are likely to contain multiple forms of fatty acid ω-1 hydroxylase, and the cDNA of one of them has been cloned in yeast cells (11). Recently, Williams and co-workers (6) described the high regioselectivity of lauric acid hydroxylation at ω-1 position by liver and kidney microsomes from untreated rainbow trout. Antibodies raised against a purified cytochrome P-450 from trout (trout $P450LM_2$), active toward aflatoxin B_1, inhibited laurate hydroxylation in both liver and kidney microsomes. Treatment of fish with β-naphthoflavone decreases the rate of laurate oxidation but strongly enhances 7-ethoxyresorufin *O*-de-ethylase (EROD) activities. Very recently, Miranda *et al.* (12) reinvestigated the catalytic activity of hepatic cytochrome P-450 from untreated trout and demonstrated that lauric acid can be hydroxylated at both ω and ω-1 positions. Each type of oxidation was carried out by a highly regiospecific P-450, designated LMC_1 (laurate ω hydroxylase) and LMC_2 (laurate ω-1 hydroxylase). Other enzyme systems hydroxylating lauric acid, from carp (13) and *Tilapia aurea* (14), have been described but metabolites have not been fully characterized.

Most of the relevant publications on monooxygenases from fish concern the oxidation of xenobiotics. Few studies have been conducted on the metabolism of endogenous substrates except for those on sex hormones. The objective of our present study was to compare the *in vitro* metabolism of fatty acids and of xenobiotics by microsomal monooxygenases from the liver of sea bass. Using lauric acid as a model substrate for monooxygenases that hydroxylate fatty acids, we have identified the reaction products by a combination of thin-layer chromatography (TLC) and reverse phase high-performance liquid chromatography (RP-HPLC) methods, and have characterized the laurate metabolites by gas chromatography/mass spectrometry (GC/MS). Laurate hydroxylase activities and the ratio of metabolites formed from fish farm sea bass and from a wild type are compared. We also report the effects of starvation, feeding and treatment with benzo(*a*)pyrene (BP) on xenobiotics metabolism and laurate oxidation from liver microsomes of farm sea bass.

MATERIALS AND METHODS

Chemicals. Lauric acid (sodium salt), NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and benzo(*a*)pyrene were purchased from Sigma-Chimie (La Verepillière, France). Radiolabeled [1-^{14}C]lauric acid (spec. act. 56 Ci/mol) was from C.E.A. (Gif/Yvette, France). *N,O-bis*(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + 1% TMCS) was from

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Abbreviations: BP, benzo(*a*)pyrene; BPMO, benzo(*a*)pyrene monooxygenase; ECOD, 7-ethoxycoumarin *O*-de-ethylase; EROD, 7-ethoxyresorufin *O*-de-ethylase; GC/MS, gas chromatography/mass spectrometry; LAH, lauric acid hydroxylase; RP-HPLC, reverse phase high-performance liquid chromatography; TLC, thin-layer chromatography.

Pierce-Europe (Oud-Beijerland, The Netherlands). Acetonitrile (HPLC grade) and silica gel plates (G60 F254, 0.25 mm) were from Merck (Darmstadt, Germany).

Fish. Sea bass (*Dicentrarchus labrax*), obtained from two local fish farms, were conditioned for 3 wk in 600-L tanks, and fed a commercial sea bass diet (Aqualim) before the experiments. Wild sea bass were caught in the Mediterranean Sea (Banyuls, France). During experiments, marine fish were placed in a glass tank containing 120 L of synthetic sea water (20g/L, 20°C) under a 12-hr light/12-hr dark cycle. Water was oxygenated and cleaned through activated charcoal filters with a pump delivering 1000 L/hr.

Sea bass from the farm "Les poissons du Soleil" (Balaruc-les-Bains, France), weighing 71 ± 6 g, were starved 48 hr and then treated with a single intraperitoneal injection (100 μL) of sterilized maize oil (control) or 100 μL of sterilized maize oil containing benzo(*a*)pyrene to give a dose of 2 mg/kg or 20 mg/kg body weight. The fish were sacrificed 14 hr after injection and hepatic microsomes were prepared.

In a second experiment, sea bass from the farm "Aquamed" (Théoule-sur-mer, France), weighing 69 ± 4 g, were divided into three groups. One group was denied food for 2 mo. The second group was fed the Aqualim diet *ad libitum* throughout the experiment. The third group was denied food for 2 mo and then fed again the Aqualim diet for 2 wk.

Microsomal preparations. Hepatic microsomes were prepared by differential centrifugation. The fish were sacrificed by severing their spinal cords. The livers (about 1 g) were immediately removed and washed with 150 mM KCl solution. The minced livers were homogenized in 5 vol (1:5, g/mL) of 250 mM sucrose/10 mM Hepes buffer (pH 7.4) using a Potter-Elvehjem homogenizer (Poly-LABO, Strasbourg, France). The homogenates were centrifuged at 12,000 × *g* for 15 min in a Beckman J2-21 centrifuge (Fullerton, CA). Resultant supernatants were centrifuged again at 105,000 × *g* for 60 min in a Beckman L5-50 ultracentrifuge. Microsomal pellets were resuspended in 2.5 mL of 250 mM sucrose/10 mM Hepes buffer (pH 7.4) containing 20% glycerol, and were stored in liquid nitrogen until assayed.

Measurement of enzyme activities. Lauric acid hydroxylase was assayed as previously described (15). The standard assay was contained in a final volume of 0.2 mL, 0.1–0.3 mg microsomal proteins, 0.1 M sodium phosphate buffer pH 7.4, 1 mM NADPH, 6.7 mM glucose-6-phosphate, 0.4 U glucose-6-phosphate dehydrogenase, 2.12 μM [1-^{14}C]lauric acid and sodium laurate to make a final concentration 100 μM. A rapid procedure was generally used to measure enzyme activity, and to analyze the compounds formed. The reaction mixture incubated at 28°C was stopped after 20 min by adding 200 μL of acetonitrile containing 0.2% acetic acid. After cooling 5–10 min on ice and a rapid centrifugation (1500 × *g*, 5 min), 50–100 μL of supernatant was injected in a RP-HPLC column or spotted on silica gel plates.

When larger quantities of metabolites were needed for GC/MS analysis, the whole procedure was scaled up tenfold. The reaction was stopped and immediately extracted twice with 10 mL of benzene/diethyl ether (9:1, v/v). After filtration over anhydrous Na_2SO_4 and drying under vacuum, samples were dissolved in a small volume of methanol and subjected to RP-HPLC or TLC analysis.

For carbon monoxide enzyme inhibition, the phosphate buffer used in the standard assay was saturated by bubbling CO for 1 hr into the buffer. Inhibition studies were performed by adding 50 and 100 μL of phosphate buffer saturated with CO to the incubation medium (final volume of 200 μL) in capped tubes. Control experiments were performed as described for the standard assay in open and capped tubes. No difference in lauric acid hydroxylase (LAH) activities was observed between open or capped tubes using these conditions.

Ethoxyresorufin *O*-dealkylase (EROD) and ethoxycoumarin *O*-dealkylase (ECOD) activities were measured according to Burke and Mayer (16) and Ullrich and Weber (17), respectively. Benzo(*a*)pyrene monooxygenase (BPMO) activity was determined by 3-hydroxybenzo(*a*)pyrene formation as described by Nebert and Gelboin (18). Assays were performed at 30°C using a fluorimetric method with a Kontron SFM 25.

All enzymatic assays were linear as a function of time and protein concentration under the conditions used for enzyme measurements.

Statistical analysis. Statistical data analysis was by Student's t-test (19).

Chromatographic conditions. RP-HPLC analyses were carried out on a 15 cm × 4.6 mm Beckman Ultrasphere ODS 5 μm C18 column as described (15). To measure radioactivity distribution, lauric acid and hydroxylated reaction products were injected either in 20 μL of methanol or directly in 50–100 μL of reaction medium containing 50% acetonitrile. Samples were developed (2 mL/min) using an initial mobile phase of acetonitrile/water/acetic acid (25/75/0.2, v/v/v) to elute the hydroxylated products. Residual lauric acid was eluted using a linear gradient (0 to 100%) of acetonitrile/water/acetic acid (80:20:0.2, v/v/v) 35 min after injection at the same flow rate for 20 min. Radioactivity of RP-HPLC effluents was monitored with a computerized on-line solid scintillation counter (Ramona-D RAYTEST, Straubenhardt, Germany).

For quantitative evaluation, 100-μL aliquots of incubation medium were spotted on silica TLC plates. The plates were developed (15 min longer than for solvent migration to the top of the plate) in a system of diethyl ether/light petroleum hydrocarbon (b.p. 40–60°C)/formic acid (70:30:1, v/v/v). After detection of the radiolabeled metabolites with a thin-layer linear analyzer (Berthold LB 511, La Garenne-Colombes, France), the areas corresponding to ω plus ω-1 hydroxylaurates (Fig. 1, peak A) and to ω-4, ω-3 and ω-2 hydroxylaurates (Fig. 1, peak B) were scraped into counting vials, and radioactivity was measured with an Intertechnique SL 4000 liquid scintillation counter (Intertechniques, Plaisir, France).

Gas chromatography/mass spectrometry. Products formed were first separated in two radioactive peaks by the TLC procedure described above. Radioactive compounds contained in peaks A and B (Fig. 1) were scraped off the plates and eluted twice from the silica with 10 mL of benzene/diethyl ether (1:1, v/v). An aliquot of each was subjected to RP-HPLC analysis (Fig. 2, B and C) with the appropriate mobile phase as described above. Prior to GC/MS analysis, the metabolites of lauric acid were methylated and silylated using a procedure described previously (15). Samples in a mixture of hexane/BSTFA (1:1, v/v) were injected (1–2 μL) in the splitless mode, and

were run on a capillary column (30 m × 0.25 μm coating thickness) packed with 1% SE 30 on Chromosorb W-AW DMCS (Supelco-France, Paris). The oven temperature was programmed from 80 to 200°C at a rate of 3°C/min. The gas chromatograph was interfaced with an LKB 9000S mass spectrometer with an LKB 2130 computer on-line system (LKB Produkter, Bromma, Sweden). Spectra were obtained in the EI mode at 70 eV.

Spectroscopic assays. Spectrophotometric hemoprotein determinations were carried out on a Shimadzu MPS 2000 instrument (Roucaire, Strasbourg, France). Microsomal amounts of cytochromes P-450 and b_5 were measured by the method of Omura and Sato (20) using the following extinction coefficients: 91 mM^{-1} cm^{-1} for the 450–490 nm absorbance difference, and 185 mM^{-1} cm^{-1} for the 424–409 nm absorbance difference, respectively. Microsomal protein concentrations were determined according to Lowry *et al.* (21) using bovine serum albumin as standard.

RESULTS

Microsomal incubations with lauric acid. As previously shown for cytochrome P-450 catalyzed hydroxylation of lauric acid, both molecular oxygen and NADPH were essential for hydroxylation of lauric acid by liver microsomes from sea bass. Carbon monoxide, which interacts with the active site of cytochrome P-450 enzymes, inhibited hydroxylation suggesting the involvement of cytochrome P-450 in both reactions. The reaction was linear up to 20 min at 28°C for protein concentrations below 380 μg of liver microsomal proteins (not shown).

The mixture of reaction products was resolved by TLC into two major radioactive peaks (Fig. 1, A and B). When compounds eluted from the TLC zone A were analyzed by RP-HPLC, two metabolites with retention times corresponding to ω-1 and ω hydroxylaurates (peaks 1 and 5, respectively) were eluted (Fig. 2, B). Elution of compounds containing zone B showed three metabolites with reten-

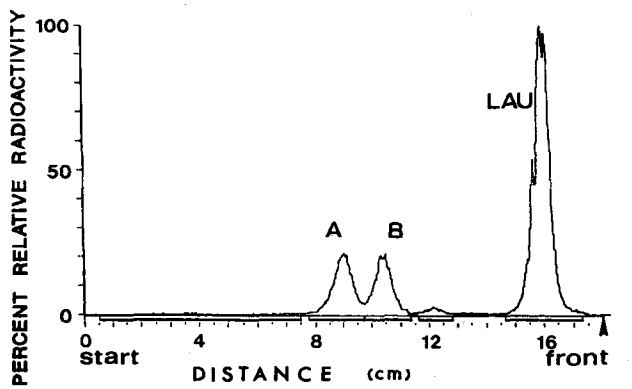

FIG. 1. TLC analysis upon microsomal incubation of [1-^{14}C]lauric acid. Radiolabeled metabolites extracted from the microsomal incubations of farm sea bass (fed throughout) with lauric acid (LAU) were subjected to TLC analysis on Silica Gel G (see Materials and Methods). Compounds in zones A (ω and ω-1 hydroxylaurate) and B (ω-2, ω-3 and ω-4 hydroxylaurate) were scraped off the plate and eluted. Aliquots were subjected to RP-HPLC (Fig. 2) and mass spectrometry.

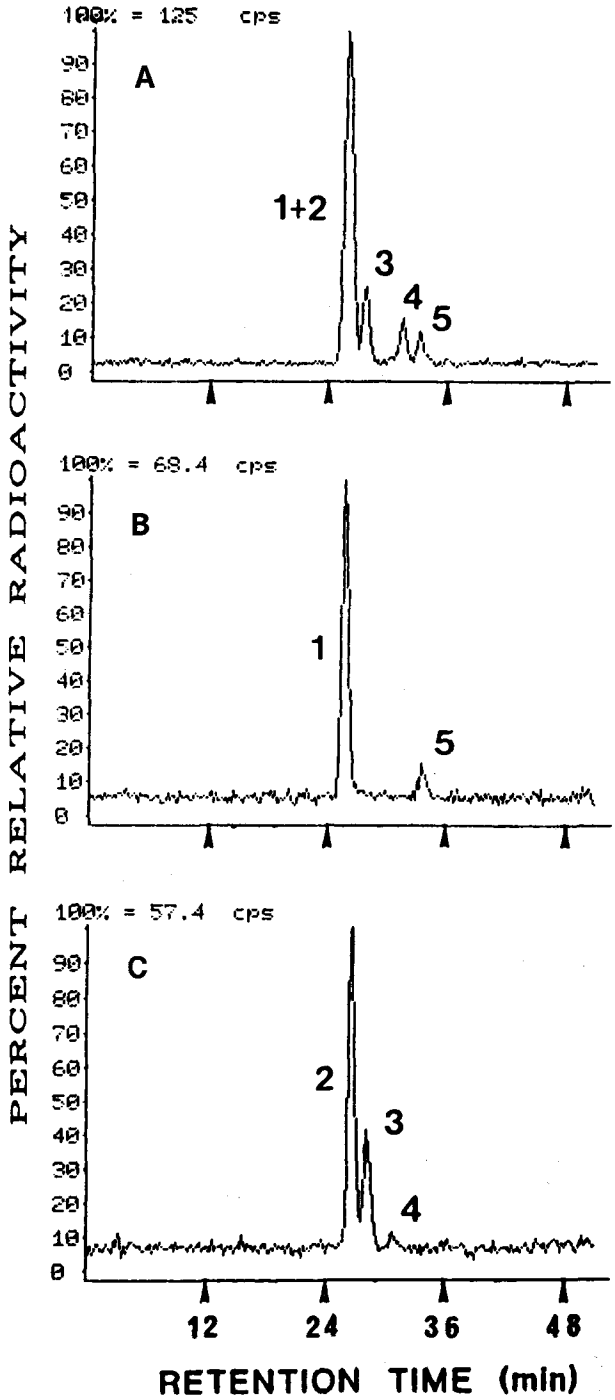

FIG. 2. RP-HPLC analysis of hydroxylated metabolites from microsomal incubations with [1-^{14}C]lauric acid. Microsomes from farm sea bass (fed throughout) were aerobically incubated with radiolabeled laurate and NADPH. (A) Total hydroxylated metabolites formed by microsomal incubation. (B) Hydroxylated metabolites eluted from zone A of TLC plate (Fig. 1). (C) Hydroxylated metabolites eluted from zone B of TLC plate (Fig. 1). Radiolabeled metabolites eluted from RP-HPLC column were: peak 1, ω-1 hydroxy-; peak 2, ω-2 hydroxy-; peak 3, ω-3 hydroxy-; peak 4, ω-4 hydroxy- and peak 5, ω hydroxylaurate. Forty min after starting analysis, a linear gradient of 80% acetonitrile was applied to elute residual substrate (not shown). Conditions of incubation, TLC and RP-HPLC analyses are described under Materials and Methods.

TABLE 1

Effects of Starvation, Feeding and Treatment with BP on Monooxygenase Activities and Contents of Cytochromes P-450 and b_5 in Hepatic Microsomes from Sea Bass[a]

Treatment	Benzo(*a*)pyrene			Diet effects			
Enzyme activity	Control (maize oil)	2 mg/kg	20 mg/kg	Control (fed throughout)	Denied food 2 mo	Denied food 2 mo and fed 2 wk	Wild fish
EROD	0.432 ± 0.079	0.349 ± 0.032 N.S.	1.383 ± 0.192[b]	0.86 ± 0.03	0.58 ± 0.08[c]	3.25 ± 0.63[b]	0.144 ± 0.027
ECOD	0.028 ± 0.003	0.033 ± 0.001 N.S.	0.054 ± 0.002[b]	0.009 ± 0.002	0.021 ± 0.003[d]	0.048 ± 0.011[b]	0.026 ± 0.006
BPMO	0.764 ± 0.052	0.902 ± 0.055 N.S.	1.742 ± 0.159[c]	6.42 ± 0.69	3.396 ± 0.210[b]	14.91 ± 3.18[c]	7.33 ± 2.61
LAH	0.705 ± 0.113	N.D.	0.500 ± 0.159 N.S.	0.272 ± 0.072	0.848 ± 0.162[b]	0.492 ± 0.093[c]	0.406 ± 0.124
P-450	0.530 ± 0.065	0.591 ± 0.140 N.S.	0.375 ± 0.139 N.S.	0.462 ± 0.086	0.605 ± 0.077 N.S.	0.651 ± 0.164 N.S.	0.468 ± 0.14
b_5	0.29 ± 0.09	0.32 ± 0.03 N.S.	0.30 ± 0.01 N.S.	0.38 ± 0.06	0.47 ± 0.09 N.S.	0.48 ± 0.09 N.S.	0.33 ± 0.14

[a]Data are expressed as means ±SD for five individuals. EROD, ECOD and LAH activities are expressed in nmol min^{-1} mg^{-1} protein and BPMO in pmol min^{-1} mg^{-1} protein. Cytochrome P-450 and cytochrome b_5 contents are in nmol mg^{-1} protein. N.S., not significant. N.D., not determined. Fish farm origin and conditions of treatments and microsomal incubations are described in Materials and Methods.

[b]Significant at $P < 0.001$.

[c]Significant at $P < 0.005$.

[d]Effects due to treatment are significant at $P < 0.05$.

tion times observed (7) for ω-2, ω-3 and ω-4 hydroxylaurates (Fig. 2, C; peaks 2-4, respectively). When an aliquot of the supernatant (containing 50% acetonitrile) from incubated microsomes was directly analyzed by RP-HPLC, only four radioactive compounds were eluted (Fig. 2, A) showing that ω-1 and ω-2 hydroxylaurates were not resolved under these RP-HPLC conditions.

Characterization and distribution of metabolites. GC/MS analyses and electron impact (70 eV) studies of the methyl ester trimethylsilyl derivatives of the reaction products formed by untreated sea bass microsomes were performed as described elsewhere (3,7). Mass spectra of the methylated and trimethylsilylated derivatives showed typical fragment ions expected from monohydroxylated fatty acids (22) including: *m/z* 287 $[M-15]^+$, *m/z* 271 $[M-31]^+$, *m/z* 255 $[M-47]^+$, *m/z* 146 $[CH_2 = C(OSi-(CH_3)_3)-OCH_3]^+$, *m/z* 159 $[(CH_3O = C(OSi-(CH_3)_3)CH = CH_2]^+$ and the base peak at *m/z* 73 (TMS) which were commonly present in all hydroxylated lauric acid metabolites. From the results of the mass spectrometry studies, peaks in Figure 2 were identified as: *m/z* (relative intensity %); peak 1, 11-hydroxydodecanoic acid (*m/z* 117 (100), *m/z* 255 (9) *m/z* 258 (10)); peak 2, 10-hydroxydodecanoic acid (*m/z* 131 (100), *m/z* 273 (41)); peak 3, 9-hydroxydodecanoic acid (*m/z* 145 (100), *m/z* 259 (36)); peak 4,8-hydroxydodecanoic acid (*m/z* 159 (60), *m/z* 245 (34)) and peak 5, 12-hydoxydodecanoic acid (*m/z* 89 (43), *m/z* 103 (45), *m/z* 255 (100), *m/z* 287 (73)). The proportion of each monohydroxylaurate formed as a percentage of the bulk of hydroxylated metabolites was calculated from peak areas to be 44.0%, 35.6%, 14.5%, 3.5% and 2.3% for 11-hydroxy-, 10-hydroxy-, 9-hydroxy-, 8-hydroxy- and 12-hydroxylaurate, respectively. These results obtained with starved or BP-treated farm fish show that starvation, which increases laurate hydroxylase activity, does not change the regioselectivity of the oxidative attack of laurate in sea bass microsomes.

Effects of fish treatments on monooxygenase activities. Hepatic microsomal mixed function oxidase activities were measured in sea bass treated with two doses of BP. After a single intraperitoneal injection with 20 mg/kg body weight, EROD, ECOD and BPMO activities were significantly induced (2-3 fold) in liver microsomes (Table 1). However, at 2 mg/kg body weight, no significant induction of these activities was observed. No significant increase of laurate hydroxylase activity was observed after BP treatment.

Fish that were not fed for two months showed a significant decrease in EROD and BPMO activities (1.5- and 2-fold respectively, Table 1). In contrast, both ECOD and laurate activities were enhanced approximately three times by starvation.

Compared to control (fed throughout), resumption of feeding results in a significant increase in EROD, ECOD and BPMO activities (3.8, 5.3 and 2.3, respectively, Table 1). By contrast, laurate hydroxylase activity decreased when compared to that of starved fish, but remained 1.8-fold higher than that of control fish.

No significant variation in the microsomal amount of cytochromes P-450 and b_5 was observed.

The levels of cytochrome P-450 enzyme activities from wild fish were similar to those of farm fish.

DISCUSSION

Little information is available concerning the metabolism of endogenous substrates in marine fish. The present work demonstrates that a microsomal fraction from sea bass catalyzes both in-chain hydroxylation of lauric acid at ω-2, ω-3 and ω-4 positions and penultimate hydroxylation at ω-1 with nearly identical efficiency. Our results further confirm that microsomes from fish liver catalyze principally ω-1 hydroxylation of lauric acid. Analysis of the regioisomeric composition of the monohydroxylated metabolites formed by microsomes showed that oxygenation at ω-1, ω-2, ω-3, and ω-4 occurred in a nearly 3:2.5:1:0.2 molar ratio. These reactions are not fish-specific because microsomes from other living organisms such as plants, fungi and bacteria produced a similar pattern of reaction products. In addition, a very low level of ω-hydroxylaurate (2-3% of total metabolites) was also detected under our experimental conditions.

Strong evidence in support of the involvement of cytochrome P-450 in laurate hydroxylation is provided by the microsomal localization of the hydroxylase, the requirement of cofactors such as O_2 and NADPH, and the inhibitory effects of carbon monoxide. C-9, C-10 and C-11 are the major targets of oxidative attack in lauric acid in sea bass liver microsomes. No evidence is available that

hydroxylation of all positions, including the very low attack at the terminal methyl end, is catalyzed by the same hydroxylase. However no difference in the molar ratio of hydroxylated metabolites from laurate was observed between liver microsomes from control, starved and BP-treated fish. Moreover, microsomes from wild sea bass produced a hydroxylaurate profile identical to that of farm sea bass. Similar reactions products, with respect to substrate specificity, have been found with reconstituted rat liver microsomes containing a constitutive cytochrome P-450 enzyme (P450 IIC_{11}) or an isoform (P450 IA_1), inducible by β-naphtoflavone. These two isoforms were able to catalyze either ω and ω-1 hydroxylation (major reaction product) of arachidonic acid and its oxidation to ω-2, ω-3 and ω-4 hydroxyarachidonate (23).

In mammals, starvation, or a diet enriched in long-chain saturated fatty acids, is known to strongly enhance fatty acid hydroxylation activities. As shown in Table 1, the starvation of sea bass results in similar effects, increasing (about 3 times) laurate hydroxylation. Resumption of feeding after fasting decreases LAH activities (Table 1); however, they remain higher than in control (fed throughout) sea bass. Cytochrome P-450-dependent oxidation at the methyl end of fatty acids has been described as the first step of a metabolic process ending with excretion of short-chain dicarboxylic acids. In contrast, the biological significance of in-chain hydroxylases, which do not fit this catabolic scheme, remains obscure, and more studies are needed on the metabolic capacity of fish P-450 to understand the physiological roles of enzymes catalyzing in-chain hydroxylations of mid-chain fatty acids.

BP treatment at 2 mg/kg had no effect on EROD, ECOD and BPMO activities in liver microsomes from sea bass. At 20 mg/kg, BP treatment resulted in a significant increase of all activities. These results are similar to those obtained by Jimenez and Burtis (24) with bluegill sunfish. In addition, our results show that the strong induction of monooxygenase activities involved in xenobiotics metabolism is not paralleled by an increase of laurate hydroxylase activities (Table 1). These results suggest that cytochrome P-450 forms involved in xenobiotics and fatty acid metabolism are regulated independently. In addition, lauric acid seems to be metabolized by P-450 forms which are not induced by BP.

Starved fish exhibited lower levels of EROD and BPMO activities than control animals, but ECOD activity was slightly enhanced. Similar alterations in liver microsomal enzyme activities, involved in xenobiotics metabolism, have been reported by Jimenez *et al.* (25) during starvation of bluegill sunfish. However in our study, when feeding of sea bass was resumed, laurate hydroxylase activity was depressed, but ECOD, EROD and BPMO activities were induced. Ankley and Blazer (26) and Ankley *et al.* (27) reported that diet can be a significant factor influencing mixed function oxidase activities in channel catfish. But, to the best of our knowledge, modification of laurate activity in starved fish has not been demonstrated before. It would therefore appear that cytochrome P-450 systems involved in laurate hydroxylation are regulated independently of those implicated in detoxification (ECOD, EROD and BPMO) of foreign compounds.

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Inhibition of Ferrous-Induced Lipid Peroxidation by Pyrimido-Pyrimidine Derivatives in Human Liver Membranes

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The effects of pyrimido-pyrimidine derivatives (dipyridamole, RA-642, and RA-233) on lipid peroxidation, using d-α-tocopherol as standard, were studied in enriched membrane fractions from human and rat hepatocytes. Equimolar concentrations of ferrous sulfate and ascorbic acid were used to induce lipid peroxidation. The amount of peroxidized lipids observed in membrane fractions from human liver was smaller than in those from rat liver. In both species, however, pyrimido-pyrimidine derivatives, except for RA-233 in rat liver, inhibited lipid peroxidation dose-dependently in the following sequence: RA-642 > dipyridamole > d-α-tocopherol RA-233.
Lipids 27, 192–194 (1992).

Oxygen-derived free radicals (ODFR) have been associated with the etiology of certain pathological processes, such as ischemia re-perfusion syndrome (1,2), diabetic vasculopathy (3), cataracts (4), and liver disorders (5). The development of drugs that prevent the formation and/or cell damage by ODFR is a fundamental objective of current research (6).

Our group reported that dipyridamole, a pyrimido-pyrimidine derivative, which acts as a vasodilator and platelet aggregation inhibitor (7), reduces the incidence of post-thrombolysis re-perfusion arrhythmias (PTRA) (8) and of renal excretion of proteins in different types of glomerulonephritis (GMN) (9); PTRA and GMN are associated with ODFR cell membrane damage. We also reported that dipyridamole inhibits lipid peroxidation induced by hydroxyl anions (HA) in cell membranes of different rat tissue (10), an effect that had been previously observed by Iuliano *et al.* (11) using chemical substrates.

The aim of the present study was to assess the antiperoxidative effect of dipyridamole in liver cell membranes from humans, as distinct from those of rats, and to compare this effect with that of two other chemical congeners, RA-233 (Mopidamole) and RA-642, using d-α-tocopherol as standard.

MATERIALS AND METHODS

Materials. Dipyridamole (2,6-*bis*-(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-d]pyrimidine) was obtained from Boehringer Ingelheim S.A. (Barcelona, Spain). RA-233 (2,6-*bis*(diethanolamino)-4-piperidinopyrimido[5,4-d]pyrimidine) and RA-642 (2,6-*bis*(2-hidroxiethyl-2-metoxiethylamino)-4,8-*bis*(diethylamino)pyrimido[5,4-d]pyrimidine) were donated by Dr. Karl Thomae (Biberach an der Riss, Germany). Malondialdehyde *bis*-diethylacetal was obtained from Aldrich-Chemie (Steinheim/Albuch, Germany), and d-α-tocopherol and all other reagents were obtained from Sigma Chemical Co (St. Louis, MO).

Preparation of membrane fractions. Enriched membrane fractions from human and rat liver were used in this study. Human liver samples (n = 6) were obtained from specimens of partial hepatectomies carried out on patients with traumatic liver injury due to traffic accidents. The normal characteristics of the liver tissue were confirmed by histopathological examination. Informed consent was obtained for all patient samples. Rat liver samples were obtained from six 3- to 4-month-old male Wistar rats.

All samples were diluted 1:10 (v/v) in buffer containing 50 mM Tris, 100 mM NaCl, 0.5 mM KCl, 3.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.55 mM KH_2PO_4, and 3.20 M sucrose (pH 7.4). The samples were minced and homogenized in a Braun potter at 600 strokes/min, then centrifuged at 1,000 × *g* for 10 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant was removed, and the pellet was diluted (1:10, v/v) in the buffer mentioned above without sucrose. Crushed ice surrounded the samples throughout the procedure.

Measurement of lipid peroxidation. The products resulting from the thiobarbituric acid reaction, most of which were malondialdehyde (MDA) reaction products, were taken as indicators of lipid peroxidation in membrane fractions. A modification of a method described by R. Zimmerman (personal communication) was followed. Briefly, membrane concentrates were diluted (1:4, v/v) in the above-mentioned buffer solution but with 20 mM Tris, and were divided into 1.7-mL aliquots. Then 0.1 mL of the buffer (in the assays without inhibitors) or 0.1 mL of different concentrations of pyrimido-pyrimidine derivatives were added. Ferrous sulfate (0.1 mL) and 0.1 mL of ascorbic acid (FeAs) in increasing equimolar concentrations were added in baseline assays (without drugs); 75 μM of FeAs was used in experiments with drugs. FeAs was used to induce lipid peroxidation *via* the formation of HA (11,12).

Test tubes were incubated at 37°C for 45 min while being continuously shaken. Blanks which contained only tissue were incubated at 4°C. Subsequently the reaction was stopped and MDA was analyzed using 1 mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid. The products used in the samples were added to the blanks. After agitation, the samples were incubated at 100°C for 15 min and then centrifuged at 1,000 × *g* for 15 min at 4°C. The amount of malondialdehyde (MDA) produced was determined by measuring the spectrophotometric absorbance of the supernatant at 532 nm.

The absorbances were compared to those of a standard of malondialdehyde *bis*-diethyl-acetal. The protein content of the samples was determined using the method described by Lowry *et al.* (13); the results are expressed in nmol of MDA/mg protein.

Statistical analyses were carried out using the Epistat program. The Student's t-test was applied for comparison

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Abbreviations: Cyt C, cytochrome C; ED-50, effective concentration, 50%; FeAs, ferrous sulfate/ascorbic acid; GMN, glomerulonephritis; HA, hydroxyl anions; IC-50, inhibitory concentration, 50%; MDA, malondialdehyde; NBT, nitroblue tetrazolium; ODFR, oxygen-derived free radicals; PTRA, post-thrombolysis re-perfusion arrhythmias.

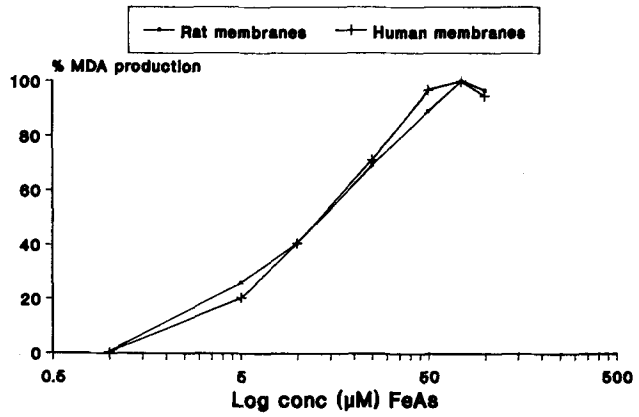

FIG. 1. Percentage of MDA production induced by different FeAs concentrations with respect to maximum MDA production obtained with 75 μM FeAs (13.5 and 18.2 nmol/mg of protein in human and rat liver, respectively).

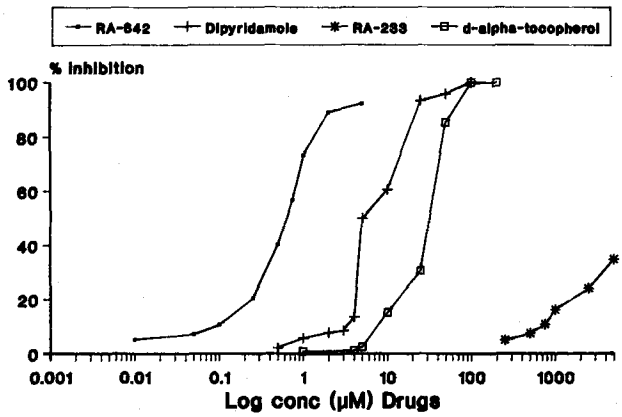

FIG. 2. Inhibition of MDA production by pyrimido-pyrimidine derivatives using 75 μM FeAs as inducer in membrane fractions from rat liver.

of nonpaired variables. Statistical significance was set at $p < 0.05$.

RESULTS

Production of MDA in cell membranes. Incubation of equimolar concentrations of FeAs with liver cell membranes caused the dose-dependent production of MDA (Fig. 1) which reached a maximum of 13.5 ± 2.3 nmol/mg of protein with human liver and of 18.2 ± 2.1 nmol/mg of protein with rat liver ($p = 0.014$). These maximum values were attained at 75 μM FeAs in both species. An FeAs concentration of 14.60 ± 1.17 μM with human liver and 14.96 ± 1.20 μM with rat liver produced 50% of the maximal MDA values. This species difference was not statistically significant ($P = 0.889$).

Inhibition of MDA production. Dipyridamole, RA-642 and d-α-tocopherol inhibited lipid peroxidation dose-dependently in rat (Fig. 2) and human liver membrane fractions (Fig. 3). In both cases, the sequence of effectiveness was RA-642 > dipyridamole > d-α-tocopherol. Peroxidation was inhibited in a concentration range of 10^{-7}M for RA-642, 10^{-6}M for dipyridamole, and 10^{-5}M for d-α-tocopherol.

When rat liver was incubated with 5 mM mopidamole, peroxidation was inhibited by only 37.8 ± 3.5%; a dose-dependent inhibition within a concentration range of 10^{-5} –10^{-3}M was observed in human liver. Table 1 shows the concentrations which inhibited MDA baseline production by 50% (IC-50).

DISCUSSION

Lipid peroxidation induced by free radicals has been associated with the development of certain liver lesions which occur upon administration of toxic substances or drugs (14) and in some liver disorders (5). Moreover, measurement of MDA after ferrous-induced peroxidation in organic samples has been proposed as a method to examine lipid peroxidation induced by HA (11,12). Our present results reflect the degree of peroxidation in human liver cell membranes induced by hydroxyl anions.

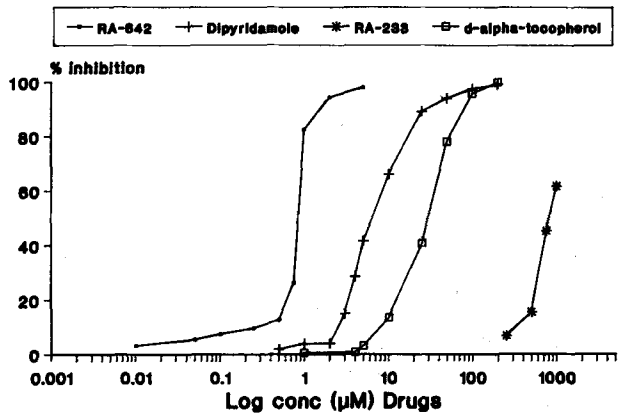

FIG. 3. Inhibition of MDA production by pryrimido-pyrimidine derivatives using 75 μM FeAs as inducer in membrane fractions from human liver.

TABLE 1

Values of 50% Inhibition (IC-50) of Ferrous-Induced Lipid Peroxidation

Compound	IC-50 (μM) Human liver membranes	IC-50 (μM) Rat liver membranes	p[a]
RA-642	0.85 ± 0.09	0.64 ± 0.05	0.0874
Dipyridamole	6.69 ± 0.77[b]	4.99 ± 0.31[c]	0.0865
d-α-Tocopherol	33.79 ± 6.83[d]	31.11 ± 5.87[e]	0.7760
Mopidamole	820 ± 60.2[f]	>5000	—

[a]Student's t-test (differences between human and rat values) [b]$p = 0.00088$, [c]$p = 0.00028$, *versus* RA-642 values [d]$p = 0.0056$, [e]$p = 0.0061$ *versus* RA-642 values, and $p = 0.0012$, $p = 0.0021$, *versus* dipyridamole values [f]$p < 0.00001$ *versus* dipyridamole, RA-642 and d-α-tocopherol values.

We observed that ferrous salts induced MDA production with similar effective concentration, 50% (EC-50) values for rat and human liver membranes. However, human liver tissue produced smaller absolute amounts of

MDA than did rat liver. The protein content was similar in both types of tissue samples (0.45 ± 0.02 mg/mL in rat liver and 0.48 ± 0.06 mg/mL in human liver, p = 0.34). According to Bosmann and Hemsworth (15), the membranes used in the present study would be expected to consist of similar morphological components in both rat and human liver. We therefore attribute observed differences in MDA production to species differences.

In the present study, we describe the inhibition of HA-induced lipid peroxidation in enriched fractions of human liver cell membranes. The results obtained with dipyridamole are consistent with data on rat tissue earlier published by our group (10), by Iuliano *et al.* (11) using a chemical fatty acid peroxidation model, and by Slater *et al.* (16) using CCl_4-poisoned rat liver microsomes. Our findings are also consistent with the results obtained by Keppler and Novacky (17) who reported that dipyridamole prevented bacterial killing by paraquat, which produces ODFR. The inhibition by dipyridamole of HA-induced lipid peroxidation is also in agreement with the inhibition by dipyridamole of proteinuria in glomerulonephritis (9), the reduction of the incidence of post-thrombolysis re-perfusion arrhythmias after a single i.v. dose of 10 mg of dipyridamole (8), and the prevention of the inhibition of prostacyclin synthesis in an experimental diabetes model (18). All these processes have been associated with ODFR production. Thus, dipyridamole appears to affect superoxide or HA formation. This was also suggested by Iuliano *et al.* (11), who showed that dipyridamole caused a decrease in the reduction of cytochrome C (Cyt C), and by our group who reported that the administration of dipyridamole decreased the reduction of nitroblue tetrazolium (NBT) produced by phenazine methosulfate in rat lens (19). Reduction of Cyt C and NBT is induced by superoxide anion formation. This would be consistent with a possible scavenger type action on superoxide anions by dipyridamole.

RA-642 has been shown to be 7.9 times more potent than dipyridamole in human tissue and 7.8 times more potent in rat tissue, while dipyridamole was 5 times more potent than d-α-tocopherol in human tissue and 6.2 times more potent in rat tissue (Table 1). We therefore conclude that RA-642 has a potent antiperoxidative effect.

Mopidamole exerts its antiperoxidative effect in human liver tissue in the mM range which is higher than the concentration required for inhibition of platelet aggregation in humans (3-5 μM) (20,21).

We conclude that in humans, pyrimido-pyrimidine derivatives, and mainly dipyridamole and RA-642, inhibit lipid peroxidation induced by ODFR. RA-642 is a new and potent antioxidant which should prove useful in the study of processes in which ODFR are involved.

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α-Tocopherol Oxidation Mediated by Superoxide Anion (O_2^-). I. Reactions in Aprotic and Protic Conditions

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The reaction of α-tocopherol (α-T) with superoxide anion (O_2^-) in both dry acetonitrile and in aqueous acetonitrile solution is described. The O_2^- was generated by the electrochemical reduction of molecular oxygen in acetonitrile, using tetrabutylammonium bromide as an electrolyte. α-T was reacted with O_2^- either in dry acetonitrile or in a 10% aqueous acetonitrile solution. In dry acetonitrile, α-T was oxidized to a very unstable primary intermediate, which was further oxidized to a secondary, more stable intermediate. The formation of the secondary intermediate depended upon the presence of molecular oxygen. This intermediate readily converted into two compounds in equimolar amounts (designated A and B). The primary, very unstable intermediate was readily reduced again to α-T by treatment with $LiAlH_4$ or ascorbic acid. However, the secondary intermediate or the stable oxidation products could not be reduced to α-T. In the 10% aqueous acetonitrile, α-T was oxidized to α-tocopheryl quinone, α-tocopherol dimer and α-tocopherol dihydroxy dimer, and an unknown compound. In the aqueous medium, no intermediates were formed by the action of O_2^-. The results of this study indicate that the reaction of α-T with O_2^- under aprotic conditions is different from that observed under protic conditions.
Lipids 27, 195–200 (1992).

The superoxide anion (O_2^-) is produced in biological systems and foods by enzymatic (1,2) or non-enzymatic reactions (3,4). It is relatively stable under anhydrous conditions, but not in aqueous media. O_2^- has been found to have detrimental effects on cells and cell constituents (5). Several studies have shown α-tocopherol (α-T) involvement in the prevention of membrane lipid peroxidation caused by O_2^-. The protective effect of α-T is thought to be due to the scavenging action of α-T which results in its oxidation by free radicals. Inconsistencies in α-tocopherol oxidation products formed under protic (aqueous) conditions and aprotic (anhydrous) conditions have previously been reported (6–10). These inconsistencies might be attributed to the instability of O_2^- under aqueous conditions and suggest that there may be differences in the reaction mechanism of α-T with O_2^- under anhydrous and aqueous conditions.

The objectives of this study were to compare the oxidation of α-T with O_2^- under protic and aprotic conditions, and to isolate the oxidation products arising under both conditions.

MATERIALS AND METHODS

Reagents. Acetonitrile (spectral grade, 0.006% water; Burdick and Jackson, Muskegon, MI), mercury (electro grade, 99.9999%; Alfa Products, Danvers, MA), ethyl acetate high-performance liquid chromatography (HPLC) grade; Fisher Scientific Company, Itasca, IL), hexane (HPLC grade; Fisher Scientific Company), isopropanol (HPLC grade; Fisher Scientific Company), tetrabutylammonium bromide (Fisher Scientific Company), α-tocopherol (Eastman Kodak Company, Rochester, NY), and oxygen (99.999%; Air Products Inc., Minneapolis, MN) were used in this study. In all experiments, doubly distilled water was used. α-Tocopheryl quinone (TQ) was synthesized and purified by the method of Eggitt and Norris (11). α-Tocopherol dihydroxy dimer (DHD) and α-tocopherol dimer (D) were synthesized according to Csallany *et al.* (12). Purification of the above compounds was carried out by the method of Ha and Csallany (13). All other chemicals used were reagent grade.

Generation of O_2^-. The O_2^- was generated by electrochemical reduction of molecular oxygen (O_2), as described by Fee and Hildenbrand (14), with slight modifications. One-tenth M tetrabutylammonium bromide (TBAB) in acetonitrile was used as a supporting electrolyte. The TBAB was recrystallized from ethyl acetate and dried overnight in a vacuum desiccator. The electrolytic cell (H-shape) contained 60 mL of the electrolyte and the compartments were separated by a sintered glass disc 1 cm in diameter. The anode was a coil of platinum wire and the cathode was a pool of mercury. O_2 was dried by passing it through both concentrated H_2SO_4 and $CaCl_2$, and bubbled continuously through the cathode compartment for 30 min with a velocity of one bubble/sec. The O_2^- was generated using a 2 mA current for 20 min. The concentration of O_2^- was determined by measuring the optical density at 250 nm, as described by Fee and Hildenbrand (14). Electrolysis was carried out in a dry-glove box (Prospect, Kansas City, MO) that was continuously purged with dry nitrogen.

Reaction conditions. Unless otherwise specified, the following conditions were used for the reaction of α-T with O_2^- in both dry acetonitrile and aqueous acetonitrile solutions. One μmole of α-T was dissolved in 100 μL acetonitrile, and was reacted with 3 mL O_2^- solution (5.2×10^{-6} M) for 1 min. The reaction was carried out in screw-capped test tubes (20 cm × 1.5 cm) in a dry-glove box. Each reaction was repeated four times. After completing each reaction, the unreacted α-T and its oxidation products were extracted 3 times with 3 mL of hexane. The combined extracts were dried over anhydrous sodium sulfate and concentrated to 3 mL under N_2 at room temperature for HPLC analysis. The HPLC analysis of α-T and its oxidation products was performed on a normal-phase column (5 μm Ultrasphere-Si, Beckman Instruments Inc., Berkeley, CA) using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) (13). The HPLC equipment used was a model 110A solvent metering pump (Beckman Instruments, Inc.) and an Altex Model 210 solvent injector equipped with a 100-μL loop (Beckman Instruments, Inc.).

Stability of O_2^-. The stability of O_2^- in the presence of water was determined. Water was added to quartz cuvet-

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Abbreviations: α-T, α-tocopherol; D, α-T dimer; DHD, α-T dihydroxy dimer; HPLC, high-performance liquid chromatography; O_2^-, superoxide anion; O.D., optical density; TBAB, tetrabutylammonium bromide; TQ, α-tocopheryl quinone.

tes (1 cm × 1 cm) containing 3 mL O_2^- solutions to produce 0, 1, 5, and 10% per volume water concentrations. Dismutation of O_2^- was determined by measuring the rate of change of optical density (O.D.) at 250 nm, using a Beckman model DU-8 Spectrophotometer equipped with a DU-8 kinetic Compuset module (Berkeley, CA).

Formation of intermediates in dry acetonitrile. To determine the formation of the very unstable primary intermediate product, α-T was reacted with the O_2^- solution. The primary intermediate and the secondary intermediate products exhibited maximum absorbance at 350 and 235 nm, respectively. The optical density of the reaction mixture was monitored at 350 nm for the formation of the primary oxidation product and 235 nm for the formation of the secondary, more stable oxidation product of α-T. The disappearance of α-tocopherol (α-T) was monitored at 292 nm as a function of time (1/3, 1, 2, 3, 4, 5, and 10 min) using a Beckman DU-8 Spectrophotometer equipped with a kinetic Compuset module. The primary intermediate product reached a maximum concentration at 20 sec. A one-minute reaction time was used for the production of the secondary intermediate.

Reduction of the primary intermediate. One-μmole samples of α-T were each reacted with 3 mL O_2^- solution for 20 sec. The reaction mixtures were reduced immediately with $LiAlH_4$ reagent according to the method of Csallany *et al.* (15) or with 0.6 g ascorbic acid for 5 min. After reduction, the reaction mixtures were extracted with hexane, the extracts were washed with distilled water and analyzed by HPLC for α-T and its oxidation products. Control samples were treated identically, except that they were not treated with the reducing agents.

Reduction of the secondary intermediate. Four one-μmole samples of α-T were each reacted with 3 mL O_2^- solution for one min and extracted with hexane. One sample was used as a control without further treatment, and the remaining samples in hexane were treated with either $LiAlH_4$, (15) ascorbic acid or 1 mL of 0.1 M HCl. The mixtures then were extracted with hexane, which was washed with distilled water, and the samples were analyzed for α-T and its oxidation products by HPLC.

Formation of stable α-T oxidation products (A and B). The formation of two stable α-T oxidation products (compounds A and B) with O_2^- in dry acetonitrile was carried out in the same way as that of the secondary intermediate product (reaction time 1 min). The unstable secondary oxidation product was converted quantitatively to compounds A and B by evaporating the solvent hexane or by washing the solvent with water. Compounds A and B were then separated by HPLC.

Reduction of the compounds A and B. Compounds A and B were treated with $LiAlH_4$ or ascorbic acid identically as described for the primary and secondary intermediates and were analyzed by HPLC for possible conversion into α-T.

Effect of molecular oxygen. A three-mL aliquot of the O_2^- solution, was reacted with one μmole α-T for 1 min and then used as control. Other aliquots of the O_2^- solution were purged with nitrogen for 6 min to remove the remaining O_2. This solution was reacted with one μmole α-T for 1 min with or without further nitrogen purge. Each reaction mixture was assayed for α-T and its oxidation products as described above.

Reaction of α-T with O_2^- in aqueous acetonitrile solution (protic conditions). Samples of one μmole α-T were reacted with 3 mL O_2^- solution for 0, 0.5, 1, 5 and 10 min in the presence of various concentrations of water (1, 5 and 10%). The samples were extracted with hexane and then analyzed by HPLC as described above.

RESULTS

Stability of O_2^-. The relationship between the O_2^- dismutation rate and water content is shown in Figure 1. The O_2^- was quite stable in absolute acetonitrile and remained at a constant level during the test period. The O_2^- was partly dismutated by the addition of a small amount of water to the solution, with a dismutation rate proportional to the water content. With a 10% water concentration, O_2^- declined nearly to zero within one minute. These results are in agreement with those of Ozawa and Hanaki (16), who observed that O_2^- dismutation was positively correlated with the water content in the reaction medium.

Reaction of α-T with O_2^- in dry acetonitrile (aprotic conditions). A very unstable primary intermediate product was formed during the reaction of α-T with O_2^- in dry acetonitrile, which was monitored by measuring the optical density changes at 350, 292, 235 nm as a function of time (Fig. 2). The rapid increase in the primary product (350 nm) was concomitant with a dramatic fall in α-T concentration (292 nm). Maximum absorption for the primary intermediate was observed at about 20 sec, after which it slowly disappeared. Its disappearance was followed by increased absorption at 235 nm (secondary inter-

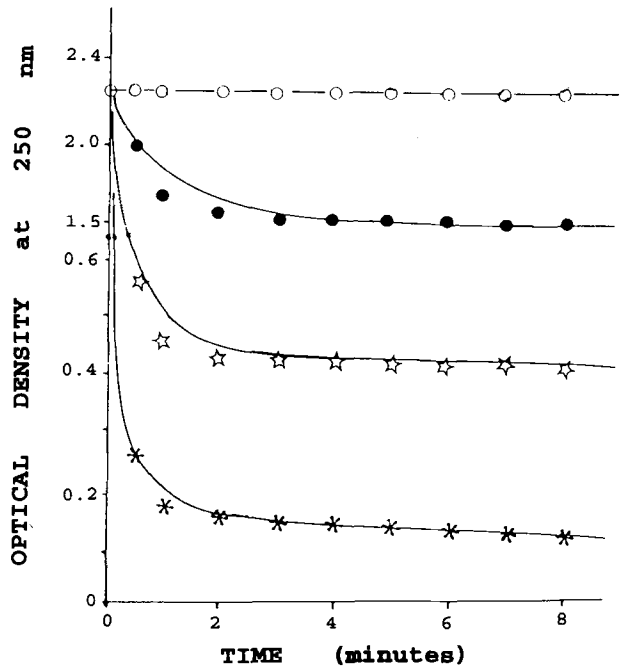

FIG. 1. Stability of O_2^- in acetonitrile containing various concentrations of water; 0 (○), 1% (●), 5% (☆), and 10% (*) water. O_2^- was generated electrochemically at 2 mA for 20 min.

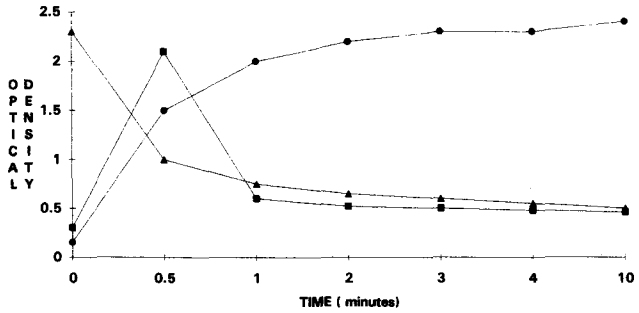

FIG. 2. Changes in optical density during the reaction of α-T with O_2^- under aprotic conditions. The primary intermediate product absorbs maximally at 350 nm (■), secondary intermediate product at 235 nm (●), and α-tocopherol at 292 nm (▲).

mediate) with a maximum intensity at 1 min. The data indicate that the reaction of α-T with O_2^- under aprotic conditions produced two distinct intermediates within 1 min. The primary product was easily reduced back to α-T by treatment with either $LiAlH_4$ or ascorbic acid, as shown in Figure 3 and Table 1. The primary intermediate product disappeared completely when the reaction mixture was treated with the reducing agents, and 91% and 58% of α-T were regenerated from the primary intermediate product when treated with $LiAlH_4$ or ascorbic acid, respectively. The limited solubility of ascorbic acid in acetonitrile may be responsible for its lower effectiveness compard to $LiAlH_4$. When no reducing agents were added, approximately 85% of the α-T converted to compounds A and B.

The α-T oxidation products produced by the reaction of α-T with O_2^- in dry acetonitrile are shown in Figure 4. Retention times were 12.8, 15.2 and 16.4 min for the α-T secondary intermediate, compound A, and compound B, respectively. The secondary product was measured at 235 nm maximum absorbance. This compound slowly converted in hexane at room temperature to compounds A and B, and also readily converted to these compounds (A and B) when washed with water or when the solvent was evaporated under nitrogen. Compounds A and B also exhibited maximum absorbance at 235 nm, and were found to be slightly more polar than the parent compound α-T. Compounds A, B, and the secondary intermediate product were not converted to α-T when treated with either $LiAlH_4$, ascorbic acid or 0.1M HCl.

The greatest conversion of α-T into the secondary intermediate product was obtained when no nitrogen was bubbled through the reaction mixture before and during the reaction of α-T with O_2^- in dry acetonitrile (Fig. 5). When the O_2^- solution was purged with nitrogen for 5 min before the reaction with α-T, considerably less secondary product formed. When the nitrogen purge was continued during the reaction of O_2^- with α-T for an additional one minute (total 6 min), the least amount of secondary product was produced. These results seem to indicate that O_2 may also be involved in α-T oxidation. Matsumoto and colleagues (17,18) observed that when O_2 was evacuated from the reaction medium, the reaction rate of an α-T model compound, 2,2,5,7,8-pentamethylchroman, with O_2^- was markedly inhibited. Nanni *et al.* (8) reported

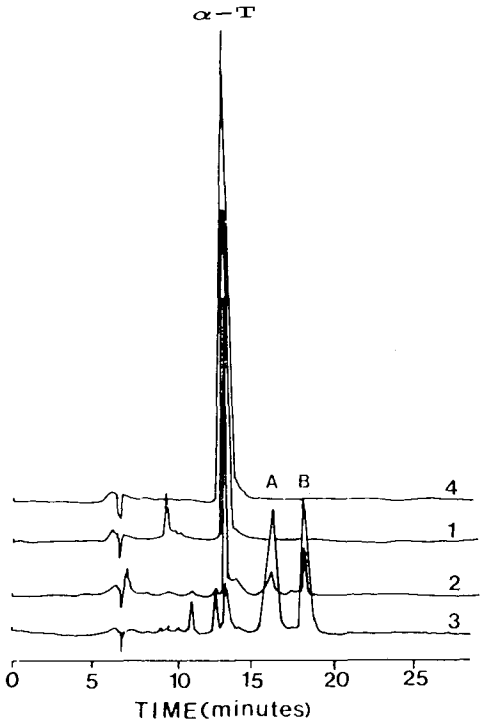

FIG. 3. HPLC tracings of the reduction products of the primary oxidation product of α-T after treatment with $LiAlH_4$ (1) or ascorbic acid (2). The primary oxidation product in the absence of reducing agent converts to compounds A and B during analysis (3). Standard α-T is shown for comparison (4). A 5 μm Ultrasphere-Si column, 250 mm × 4.6 mm i.d. was eluted with hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) at a flow rate of 0.4 mL/min. Detection was at 234 nm for compounds A and B and 292 nm for α-T; the sensitivity was 0.05 AUFS at both wavelengths.

TABLE 1

Reduction of Primary Intermediate[a]

Treatment	α-T	Compound A	Compound B
Blank[b]	9.3	0.0	0.0
Control[c]	1.4[d]	3.2	3.5
$LiAlH_4$	8.6	0.0	0.0
Ascorbic acid	6.6[d]	0.8	1.1

[a]Values are in μg and represent the average of 4 repeated experiments with triplicate injections per experiment.

[b]The blank contained α-T but no O_2^- nor reducing agent.

[c]The control contained the primary intermediate without reducing agent.

[d]Differs from blank, (Tukey's w significance test), $p < 0.05$.

that O_2 may be produced during the reaction of α-T with O_2^- and may be involved in α-T oxidation reactions as well.

Reaction of α-T with O_2^- in an aqueous acetonitrile solution (protic conditions). The disappearance of α-T was measured during the reaction with O_2^- in the presence of various amounts of water (1, 5 and 10%) in acetonitrile over a period of time and compared with a control α-T sample which contained no O_2^- and water (Fig. 6). In the presence of 1% of water (1 min reaction time), α-T quickly disappeared and produced an intermediate similar to that

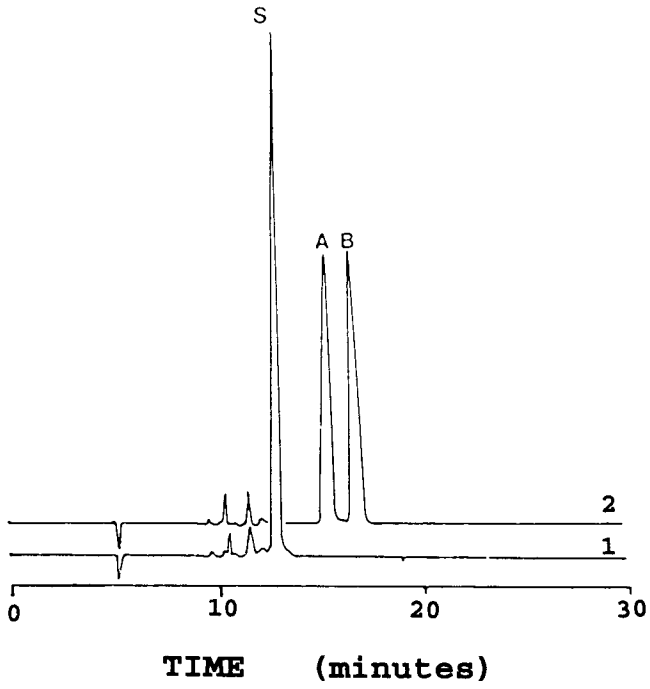

FIG. 4. Typical HPLC tracings of α-tocopherol oxidation products obtained from the reaction of α-tocopherol oxidation products obtained from the reaction of α-T with O_2^- under aprotic conditions. Chromatogram 1; secondary intermediate, S. Chromatogram 2; stable end-products compound A, A and compound B, B.

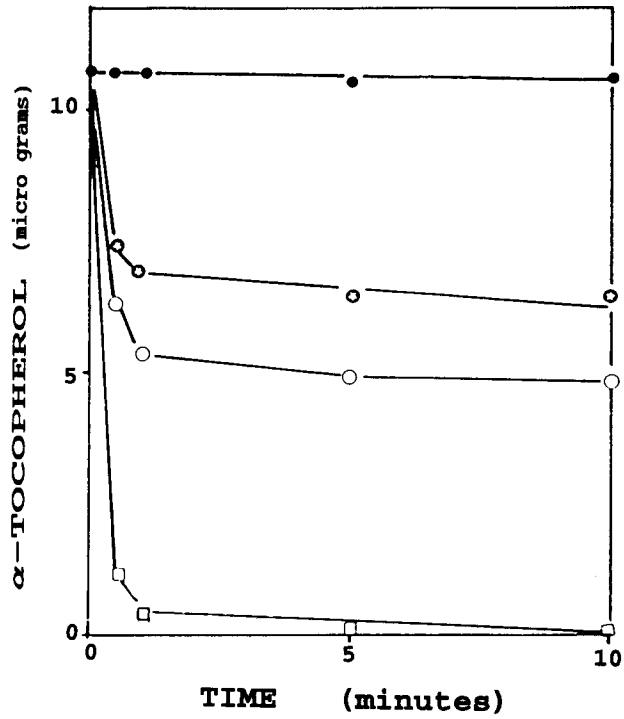

FIG. 6. Disappearance of α-T from the reaction mixture of α-T with O_2^- in the presence of various concentrations of water: 1% water (○), 5% water (○), 10% water (□) and 0% water and no O_2^- (●).

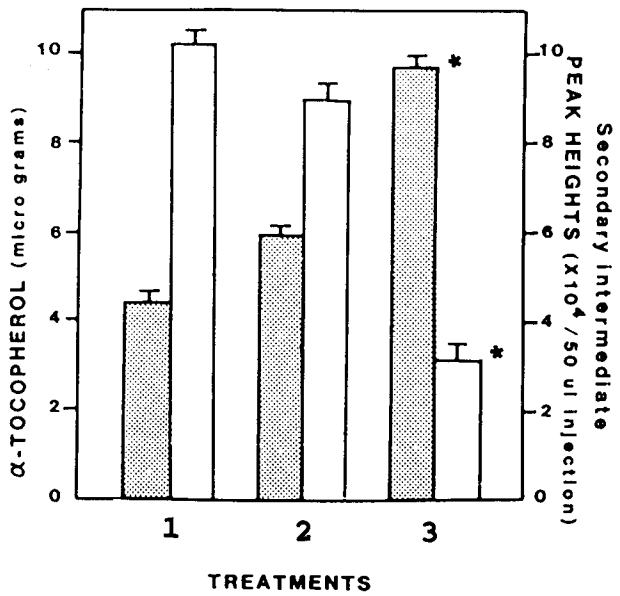

FIG. 5. Effects of N_2 purge on the reaction of α-T with O_2^- in dry acetonitrile. Treatments: 1, no nitrogen purge; 2, 5 min nitrogen purge before reaction; 3, nitrogen purge 5 min before reaction and 1 min during reaction. Shaded bars represent α-T and open bars represent secondary intermediate product. * = Differ statistically, Tukey's w significant test, $X < = 0.05$.

seen with the reaction of O_2^- itself. This result indicates that a 1% water concentration in acetonitrile is not sufficient to dismutate O_2^-. The higher reaction rate, in 10% water than in 5% water, suggests that 10% water in acetonitrile was more effective in dismutating O_2^- under the experimental conditions used. A typical HPLC separation of the products of the reaction of α-T with O_2^- in a 10% aqueous acetonitrile solution (Ultrasphere-Si column) is shown in Figure 7. Four α-T oxidation products were isolated, and three of them were identified as α-T dimer (D), α-T dihydroxy dimer (DHD) and α-T quinone (TQ) by both co-chromatography and ultraviolet spectrometry. Co-chromatography of the samples spiked with pure D, DHD and TQ did not change retention times and peak shapes when compared to the pure compounds alone. Peak heights and areas were increased proportionately to the amounts of standards added. The ultra-violet spectra of these compounds in hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) were identical to their corresponding standards (Table 2). Hence, the products of the oxidation of α-T with O_2^- were different in anhydrous acetonitrile (Fig. 4) and in the presence of 10% water in acetonitrile.

DISCUSSION

Two unstable α-T intermediate products were produced during the reaction of α-T with O_2^- in dry acetonitrile. The first intermediate was found to be very unstable (20 sec) and converted readily to the second intermediate. The

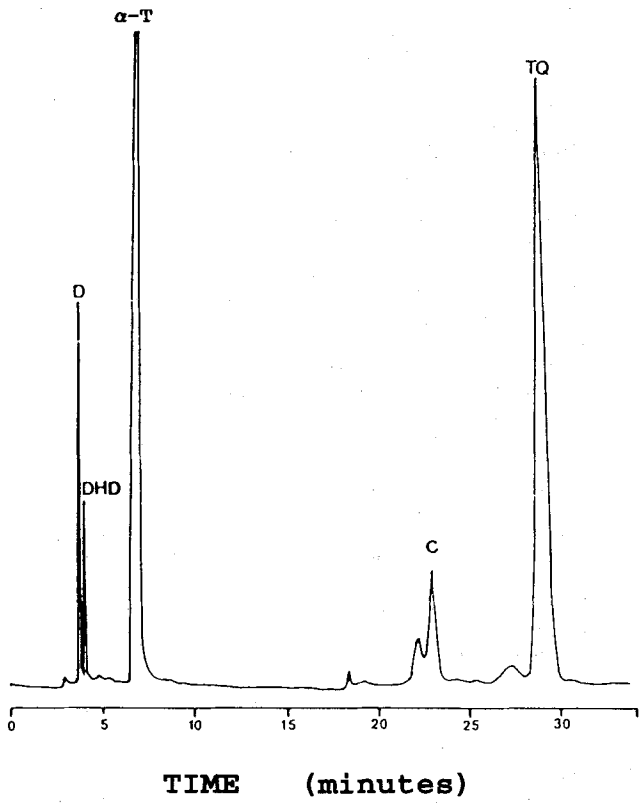

FIG. 7. A typical HPLC tracings of α-T oxidation products produced by the reaction of α-T with O_2^- in a 10% aqueous solution. The identified peaks are: D, α-T dimer; DHD, α-T dihydroxy dimer; α-T, α-tocopherol; TQ, α-tocopheryl quinone; and C, unknown compound. The HPLC conditions were as described for Figure 3.

TABLE 2

Adsorption Maxima[a] of Standards and Isolated α-Tocopherol Oxidation Products from the Reactions of α-Tocopherol with O_2^- in the Presence of 10% water

Compounds	Standards (nm)	Isolated compounds (nm)
α-Tocopherol dimer	298.5	298.6
α-Tocopherol dihydroxy dimer	291.2	291.0
α-Tocopherol	292.0	291.8
α-Tocopheryl quinone	268.0	267.8

[a]Maxima were determined in the hexane/chloroform/isopropanol (95:45:0.5, v/v/v) mixture used as mobile phase in HPLC.

secondary intermediate was more stable than the first intermediate, but also converted in about equal quantities of two stable end products, compounds A and B. The primary intermediate product was easily reconverted to α-T by treatment with $LiAlH_4$ or ascorbic acid. It may be concluded that the primary intermediate product is an α-tocopheroxy radical or an α-tocopheroxy anion. Ozawa and Hanaki (10,16,19) observed yellow color development during the reaction of an α-tocopherol model compound with O_2^-, and identified this compound as an α-chromanoxy radical by electron spin resonance (10,16,19). However, the chromanoxy radical seemed to be more stable than the primary intermediate product of α-T produced in the present experiments. According to Nanni *et al.* (8) the possibility exists for the formation of an α-tocopheroxy anion by the action of O_2^-. It has also been suggested that, in the presence of glutathione or ascorbic acid, α-T could be regenerated after being oxidized to an α-tocopheroxy radical by O_2^- (9,20). However, none of these studies provided direct evidence that α-T was the end product of the regeneration procedures. One of the interesting aspects of the present study is the demonstration of α-T regeneration from the primary intermediate product which was produced by the reaction of α-T with O_2^- in an aprotic condition. When O_2^- is produced by membrane-bound enzymes such as NADPH reductase (2,21) or by the H_2O_2 disproportionation reaction (22), α-T acts as a scavenger. If an α-tocopheroxy anion is formed by O_2^-, it could be reduced back to α-T in the presence of ascorbic acid, making α-T again available for scavenging action.

The secondary intermediate product (absorption maximum 273 nm) could not be converted to α-T by treatment with $LiAlH_4$ or ascorbic acid. It has been reported that α-tocopherone (absorption maximum 240 nm in methanol) produced from the reaction of α-T with O_2^- (20) or free radicals (3) can be reduced again to α-T by treatment with reducing agents, and can be oxidized to α-tocopheryl quinone by treatment with acid (23).

It is concluded that the secondary intermediate produced by the reaction of α-T with O_2^- under aprotic conditions is not identical to α-tocopherone because the secondary intermediate thus produced is not convertible to α-T by reducing agents nor is the secondary intermediate oxidized to α-tocopheryl quinone in the presence of acid. Production of the secondary intermediate product required O_2 in the reaction media (Fig. 5). The secondary intermediate product converted readily to equal amounts of compounds A and B in the presence of water or during sample handling. Compounds A and B were stable end products of the reaction of O_2^- under aprotic conditions.

The first step of α-T reaction with O_2^- in the aprotic media resulted in the formation of the primary intermediate product. If O_2^- in an aprotic solution acts as a Bronsted base, then phenolic compounds such as α-T (8,17,24) could be converted to α-tocopheroxy anions. The second step is the oxidation of the primary intermediate to the secondary intermediate product, mediated by the presence of O_2. The involvement of O_2 in α-T oxidation has been observed by several other investigators (8,24,25). Depending upon the reaction conditions, α-T was selectively oxidized (especially in the presence of base). The final step in the oxidation of α-T is the conversion of the secondary intermediate into the stable end products A and B.

By contrast, when α-T was reacted with O_2^- in a 10% aqueous acetonitrile solution, neither the primary intermediate product, the secondary intermediate product, nor compounds A and B were formed, rather, α-tocopherol dimer (D), α-tocopherol dihydroxy dimer (DHD), α-tocopheryl quinone (TQ) and an unknown compound were formed instead. The principal fate of O_2^- in an aqueous solution is its dismutation which results in the formation of hydroxy radical and/or singlet oxygen (5,22,26–28). Dimer, DHD, α-T trimer, and TQ are formed by the reaction of α-T with free radicals (12,23,29), in addition, TQ may be formed by reaction with singlet oxygen (30,31).

It has also been reported that TQ is produced by the reaction of α-T with O_2^- generated from the xanthine oxidase system (9). However, O_2^- dismutation must have occurred in that system in the presence of water. Therefore, TQ formation may be attributable to the formation of hydroxy free radicals. In the present experiments, α-tocopheryl quinone was not found to be produced by the direct action of O_2^-.

In summary, when α-T was reacted with O_2^- in dry acetonitrile, a very unstable intermediate product was formed. This compound could easily be converted into α-T by treatment with either $LiAlH_4$ or ascorbic acid. The primary intermediate product changed into the secondary product which was isolated by HPLC. The secondary product then converted in the presence of oxygen into two stable end products. When α-T reacted with O_2^- in a 10% aqueous acetonitrile solution, α-T dimer, α-T dihydroxy dimer, α-T quinone and an unknown compound were produced. The present experiments demonstrate that the reaction of α-T with O_2^- under aprotic conditions produces completely different oxidation products than does the reaction under protic conditions.

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α-Tocopherol Oxidation Mediated by Superoxide Anion (O_2^-). II. Identification of the Stable α-Tocopherol Oxidation Products

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The present paper describes the identification of two stable end products of α-tocopherol oxidation that were previously detected among the products of the reaction of α-tocopherol with superoxide anion (O_2^-) under aprotic conditions. One compound, previously designated compound A, was identified as *trans*-7-hydroxy-*trans*-8,8a-epoxy-α-tocopherone, and the other, designated compound B, was identified as *cis*-7-hydroxy-*cis*-8,8a-epoxy-α-tocopherone. It was also observed that under protic conditions (10% water in acetonitrile) the reaction of α-tocopherol with O_2^- did not produce compounds A and B, but rather α-tocopheryl quinone, α-tocopherol dimer, α-tocopherol dihydroxy dimer, and the previously designated compound C. Compound C was identified in the present study as α-tocopheryl-quinone-2,3-epoxide.
Lipids 27, 201-205 (1992).

Following the first report by Fridovich (1) on the production of superoxide anion (O_2^-) in biological systems, several laboratories became interested in the reaction of α-tocopherol (α-T) with O_2^- under various conditions (2-11). Recent studies from our laboratory (12) showed that the reaction of α-T with O_2^- proceeded by different mechanisms under aprotic and protic conditions. By use of high-performance liquid chromatography (HPLC) (12), two stable unidentified compounds (A and B) were isolated from the reaction products of α-T with O_2^- under aprotic conditions, while α-tocopheryl quinone, α-tocopherol dimer, α-tocopherol dihydroxy dimer and an unidentified compound C were isolated from the reaction products of α-T with O_2^- under protic conditions (13). In the present paper, the identification of compounds A, B and C is reported.

MATERIALS AND METHODS

Reagents. Acetonitrile (spectral grade, 0.0006% water; Burdick and Jackson, Muskegon, MI), mercury (electro grade 99.999%; Alfa Products, Danvers, MA), ethyl acetate (HPLC grade; Fisher Scientific Company, Itasca, IL), hexane (HPLC grade; Fisher Scientific Company), isopropanol (HPLC grade; Fisher Scientific Company), tetrabutylammonium bromide (Fisher Scientific Company), α-tocopherol (Eastman Kodak Company, Rochester, NY), and oxygen (99.999%; Air Products, Inc., Minneapolis, MN) were used in this study. In all experiments, doubly distilled water was used. All other chemicals were reagent grade.

Instrumentation. HPLC separation of α-tocopherol (α-T) and its oxidation products was performed on a 5 μm Ultrasphere-Si, 250 mm × 4.6 mm, i.d., column (Beckman Instruments Inc., Berkeley, CA), using a mobile phase of hexane/chloroform/isopropanol (95:4.5:0.5, v/v/v) as described previously (12,13). Ultraviolet (UV) spectra were recorded with a Beckman model DU-8 spectrophotometer. Mass spectral (MS) analyses were carried out with a Kratos MS-25 instrument (Manchester, England) equipped with a Data General Eclipse s/120 data system. For electron impact mass spectroscopy (EI-MS), the ion source temperature was 230°C, and the ionizing voltage was 70 eV. For chemical ionization mass spectroscopy (CI-MS), a 220 eV ionization voltage with isobutane as the reagent gas was used. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained at 300 MHz with a Nicolet 300 WB Fourier transform (FT) NMR spectrometer equipped with a Nicolet 1280 data system (Shamburg, IL). Infrared (IR) spectra were recorded using a Nicolet 60S FT IR spectrometer equipped with a triglycine sulfate (TGS) detector.

Reaction of α-T with O_2^- (aprotic conditions). One μmole of α-T was dissolved in 100 μL dry acetonitrile and reacted with 3 mL of O_2^- solution (5.2×10^{-6}M) for one minute. The reaction mixture was extracted three times with 3 mL of hexane, the combined extracts were washed three times with 3 mL of water and dried over anhydrous sodium sulfate. The dried hexane extract was then concentrated to about 3 mL under a stream of nitrogen.

Reaction of α-T with O_2^- (protic conditions). The procedure was the same as described above, except that the 3 mL of O_2^- solution contained 10% water (v/v). Reaction conditions have been described in detail previously (13).

High-performance liquid chromatography (HPLC). The concentrated hexane extracts were analyzed by HPLC as described previously (12,13).

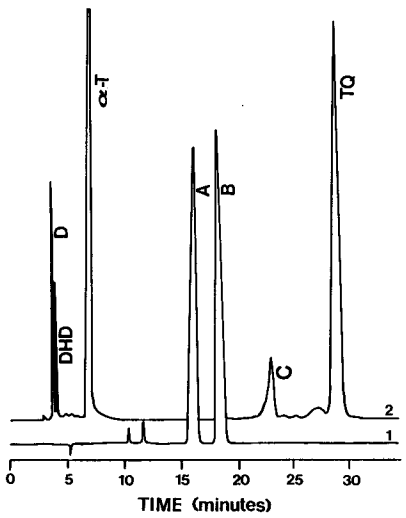

FIG. 1. HPLC separation of compounds A and B (tracing 1). HPLC separation of α-tocopherol dimer (D), α-tocopherol dihydroxy dimer (DHD), α-tocopherol (α-T), compound C (C), and α-tocopheryl quinone (TQ) (tracing 2).

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Abbreviations: $CDCl_3$, deuterated chloroform; CI-MS, chemical ionization mass spectroscopy; EI-MS, electron impact mass spectroscopy; FT, Fourier transform; HPLC, high-performance liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; O_2^-, superoxide anion; TGS, triglycine sulfate; α-T, α-tocopherol; UV, ultraviolet.

RESULTS AND DISCUSSION

The reaction of α-T with O_2^- in dry acetonitrile produced two unstable intermediate and two stable end products (13). The stable end products (Compounds A and B) were isolated and purified by HPLC (Fig. 1, Chromatogram 1). Several fractions containing these compounds were individually collected, combined, and dried under nitrogen. The residues were redissolved in methanol for UV and MS, in carbontetrachloride for IR, and in deuterated chloroform ($CDCl_3$) for NMR analyses.

The ultraviolet absorption maxima of compounds A and B were found to be at 235 nm and 234 nm and the molar absorptivities (ε) were 3060 and 3100, respectively, indicating that both compounds A and B contained a conjugated enone system (5,14,15). Infrared spectral analyses showed the presence of a hydroxyl group (3586 cm^{-1}) and a carbonyl group (1720 cm^{-1}) in both compounds.

CI-MS data of compounds A and B are shown in Figure 2. The molecular weight of both compounds was 462 daltons. Ion ($M^+ + 1$) appeared at *m/z* 463 indicating the addition of two atoms of oxygen to α-T. The EI-MS of these compounds were almost identical (Fig. 3); their distinct features were not sufficient to pinpoint structural differ-

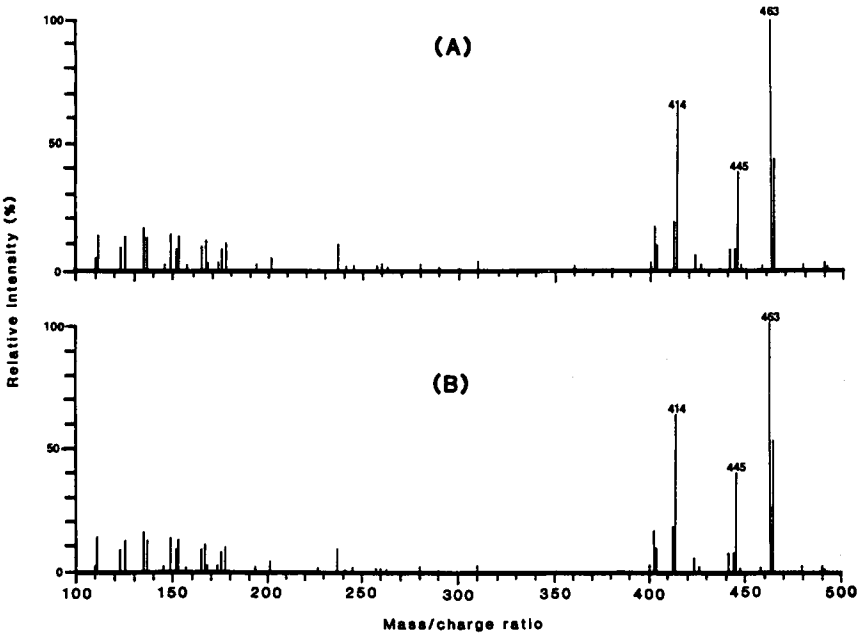

FIG. 2. CI-MS of compounds A and B.

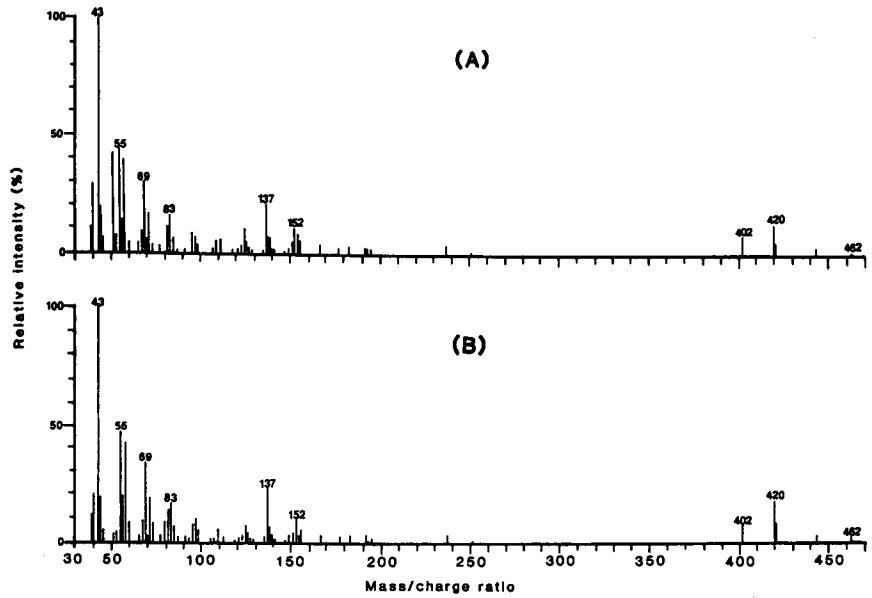

FIG. 3. EI-MS of compounds A and B.

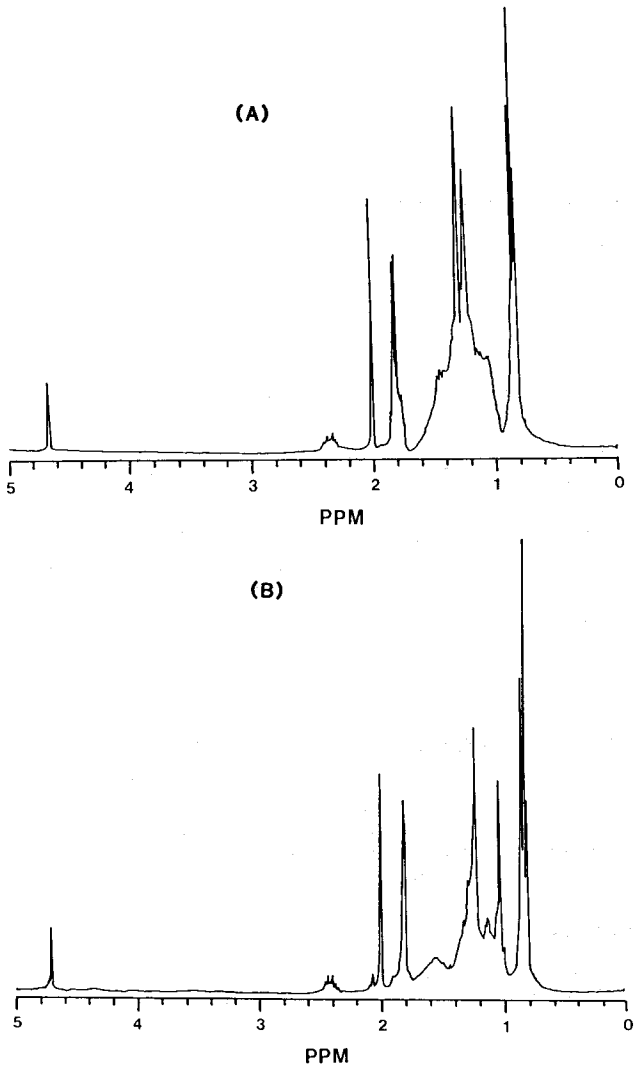

FIG. 4. NMR spectra of compounds A and B in $CDCl_3$.

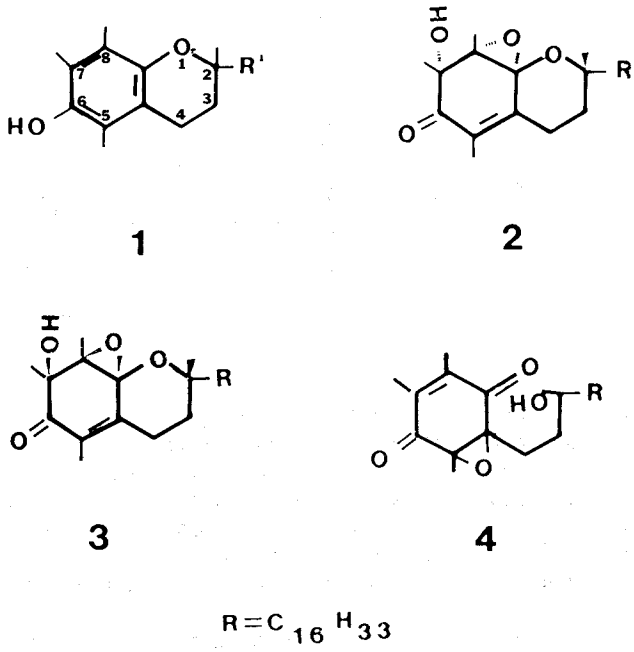

FIG. 5. Structures of α-tocopherol (1); compound A, *trans*-7-hydroxy-*trans*-8,8a-epoxy-α-tocopherone (2); compound B, *cis*-7-hydroxy-*cis*-8,8a-epoxy-α-tocopherone (3); compound C, α-tocopheryl-quinone-2,3-epoxide (4).

ences. Based on UV, IR, CI-MS and EI-MS analysis, it was concluded that both compounds A and B have a chromane ring structure, and contain an epoxide group and a hydroxyl group, as well as a ketone group at the C-6 position.

The NMR spectra for compounds A and B are shown in Figure 4. The signal assigned to a hydroxyl proton was observed at 4.70 ppm in the spectra of both compounds. Substantial differences in the NMR spectra were observed in the 1–3 ppm region. The methyl protons assigned to C-2 of compound A appeared at 1.04 ppm, those of compound B appeared at 1.30 ppm. The other proton signals of the two compounds were quite similar. The hydroxyl protons of compound A or B were shielded, indicating that the hydroxyl group may be located between a ketone group (C-6) and an epoxide group on the phenol ring thus maintaining an enone system. The chemical shift of the hydroxyl group in 2,2′,5,7,8-pentamethyl-5-hydroxy-α-chromanone was observed by Matsuo *et al.* at 3.5 ppm (5). Matsumoto and Matsuo (6) reported that the chemical shift of the hydroxyl group in 2,2′,5,7,8-pentamethyl-4a,5-7;8-diepoxy-8a-hydroxy-α-chromanone appeared at 3.05 ppm. Both 8a- and C-5 positions, therefore, can be ruled out as the hydroxyl positions in compounds A and B. Hence, C-7 appears to be the likely position for the hydroxyl group, and 8 and 8a for the epoxide group in both compounds. Compounds A and B seem to be isomers of 7-hydroxy-8,8a-epoxy-α-tocopherone. The compound contains at least three asymmetric centers at the C(2)-CH_3, C(7)-OH, and C(8),C(8a)-epoxide positions, respectively (Fig. 5, compound 2). This appears to indicate the possible existence of eight stereoisomers; however, only four isomers are formed due to ring fusion (*cis* and *trans* ring). Grams *et al.* (16) have shown that a model compound 4a, 5-epoxy-8a-methoxy-α-tocopherone exhibited *cis*-methoxy, *cis*-epoxy and *trans*-epoxy isomers to the C(2)-methyl group with a *cis* ring fusion; and *trans*-methoxy, *cis*-epoxy and *cis*-methoxy, *trans*-epoxy isomers to the C(2)-methyl groups with *trans*-ring fusion. Since the hydroxyl groups of both compounds A and B appeared at 4.70 ppm, the configuration of these compounds could be either *trans*-hydroxyl, *trans*-epoxy or *cis*-hydroxyl, *cis*-epoxy to the C(2)-CH_3 belonging to the *cis* ring fusion described above. The protons at 1.04 ppm of the C(2)-methyl group of compound A were deshielded compared to those at 1.30 ppm of compound B. Hence, compound A is proposed to be *trans*-7-hydroxy-*trans*-8,8a-epoxy-α-tocopherone (Fig. 5, compound 2) whereas compound B is proposed to be *cis*-7-hydroxy-*cis*-8,8a-epoxy-α-tocopherone (Fig. 5, compound 3).

The reaction of α-T with O_2^- under protic conditions (10% water in acetonitrile) resulted in the formation of α-tocopheryl quinone, α-tocopherol dimer, α-tocopherol dihy-

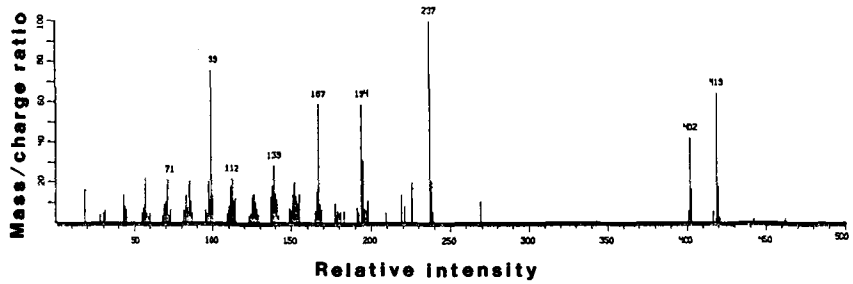

FIG. 6. EI-MS of α-tocopherol quinone-2,3-epoxide isolated by HPLC.

droxy dimer and an unidentified compound designated to be compound C (13). Figure 1 (Chromatogram 2) illustrates a typical HPLC separation of these compounds. To obtain enough material for identification, the middle portions of peak C from several HPLC separations were combined. The pooled fractions were dried under nitrogen, redissolved in methanol, and used for UV and MS analyses (Fig. 6).

An ultraviolet absorption maximum was found at 274 nm, indicating the presence of a dione conjugated double-bond system and suggesting that the chromane ring was opened. EI-MS spectra showed peaks at *m/z* 462 (M^+, 3%), 444 ($M^+ - H_2O$, 5%), 419 ($M^+ - CH_3 - CO$, 65%), and 237 ($M^+ - C_{16}H_{33}$, 100%) (Fig. 6) indicating that this compound contains one hydroxyl group (444, $M^+ - H_2O$) and a carbonyl group (419, $M - CH_3 - CO$). Furthermore, the 237 *m/z* fragment (M − R sidechain $C_{16}H_{33}$) indicates that the phenolic part of the chroman ring contains one additional oxygen. The EI-MS of α-tocopheryl quinone shows a fragment at *m/z* 221 ($M - C_{16}H_{33}$) which contains one less atom of oxygen than does compound C (16,17). Based on the data in both spectra, compound C was identified as an α-tocopheryl quinone-2,3-epoxide (Fig. 5, compound 4). The UV and MS spectral data for this compound are in agreement with the data of Grams and Inglett (17) reported for α-tocopheryl quinone-2,3-epoxide.

The principal fate of O_2^- in aqueous solution is its dismutation *via* disproportionation reactions resulting in the formation of a hydroxyl free radical (•OH) (18–21). The formation of singlet oxygen O_2^- under the above conditions remains to be controversial. Active oxygen species seem to be responsible for the formation of α-tocopherol dimer, α-tocopherol dihydroxy dimer, α-tocopheryl quinone and α-tocopheryl quinone-2,3-epoxide. Several investigators have observed the formation of α-tocopheryl quinone, dimer, dihydroxy dimer and trimer of α-tocopherol, by the reaction of α-T with hydroxy free radicals. α-Tocopheryl quinone and α-tocopheryl quinone-2,3-epoxide may be formed from the reaction of α-T with singlet oxygen (22–25). However, if O_2^- dismutation results in hydroperoxy radical (HOO•) formation, this active oxygen species could be responsible for the reaction products formed under protic conditions.

In summary, the reaction of α-T with O_2^- under anhydrous (aprotic) conditions resulted in the formation of an unstable intermediate which was converted to a somewhat more stable second intermediate (13) that, in the presence of molecular oxygen, is proposed to convert to *trans*-7-hydroxy-*trans*-8,8a-epoxy-α-tocopherone and *cis*-7-hydroxy-*cis*-8,8a-epoxy-α-tocopherone. The results are in good agreement with the findings of Matsumoto and Matsuo (4) who isolated hydroxy chromanones after reacting α-tocopherol with potassium superoxide in tetrahydrofuran. In the presence of water (protic conditions), O_2^- dismutates to active oxygen species which lead to the formation of α-tocopheryl quinone, α-tocopherol dimer, α-tocopherol dihydroxy dimer and α-tocopheryl quinone 2,3-epoxide. The present results demonstrate the difference in the reaction of α-T with O_2^- under anhydrous conditions and in the presence of water.

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METHOD

A Sensitive and Specific Radioimmunoassay for Platelet-Activating Factor[1]

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A platelet-activating factor (PAF) analog with a reactive ω-aldehyde group at the *sn*-1 position was synthesized. The hapten-thyroglobulin conjugate was used to immunize rabbits to produce specific antibodies to PAF. The purified immunoglobulin G (IgG) fraction was found to bind stereospecifically to tritiated PAF and to crossreact minimally with lysoPAF, plasmalogens, and other phospholipids. The radioimmunoassay detected as little as 20 pg of PAF per assay tube and was used to explore agonist-induced synthesis of PAF in rabbit neutrophils. Calcium ionophore A23187 at 1 μM induced PAF synthesis peaking at 2 min and reaching basal levels after 5 min. *N*-Formyl-Met-Leu-Phe (FMLP) at 0.1 μM also stimulated rapid synthesis and degradation of PAF with a peak at 5 min. Both A23187 and FMLP stimulated PAF synthesis in a dose-dependent manner. The radioimmunoassay should be applicable to the quantitation of PAF in biological samples.
Lipids 27, 206–208 (1992).

Platelet-activating factor (PAF) is a potent phospholipid mediator with a wide spectrum of biological activities, including platelet aggregation and secretion, neutrophil aggregation and chemotaxis, hypotension, bronchoconstriction, and vascular permeability (1). The fact that the naturally occurring stereoisomer (R), but not the (S) isomer is effective in stimulating platelets and neutrophils suggests the involvement of a specific PAF receptor (2). PAF is capable of triggering biological effects at concentrations as low as 0.1 nM or less indicating that endogenous production of this potent mediator by activated cells can be extremely low.

Quantitation of PAF primarily relies on its ability to trigger rabbit platelet aggregation or labeled serotonin secretion (3). The bioassays, however, have an inherent variability as even the manner in which the platelets and assay samples are prepared can affect the results. Therefore, the development of a specific, sensitive and reproducible PAF assay, such as a radioimmunoassay, for PAF is urgently needed.

We describe here the conjugation of a novel PAF analog to thyroglobulin, and the production of specific PAF antibodies. The antibodies served as the basis for developing a radioimmunoassay. The biosynthesis of PAF in rabbit neutrophils in response to stimulation by calcium ionophore and chemotactic peptide was followed using the radioimmunoassay.

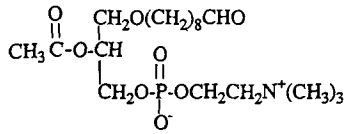
[1]Based on a paper originally presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed.

Abbreviations: CPL, choline plasmalogen; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; IC_{50}, 50% maximal binding; IgG, immunoglobulin G; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; lysoPAF, lyso platelet-activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetal-*sn*-glycero-3-phosphocholine; RIA, radioimmunoassay.

MATERIALS AND METHODS

Materials. PAF, lysoPAF, *rac*-PAF, A23187, *N*-formyl-Met-Leu-Phe (FMLP), bovine thyroglobulin, bovine serum albumin, sodium cyanoborohydride, and complete and incomplete Freund adjuvant were obtained from Sigma (St. Louis, MO). Other phospholipids were purchased from Serdary Research Laboratories (London, Ontario, Canada). The Affi-Gel protein A MAPS II Kit was supplied by Bio-Rad (Richmond, CA). Polyethylene glycol 6000 was obtained from Union Carbide (New York, NY). Goat anti-rabbit IgG was supplied by Calbiochem (San Diego, CA). New Zealand white rabbits were purchased from a local farm. 1-*O*-[Alkyl-1′,2′-^{3}H]PAF (40–45 Ci/mmol) was purchased from DuPont-NEN (Boston, MA). The PAF analog 1-*O*-(ω-nonanal)-2-acetyl-*sn*-glycero-3-phosphocholine—compound 1 (Scheme 1) was synthesized by a 10-step procedure reported elsewhere (4).



SCHEME 1

Production and purification of PAF antibodies. Conjugation and immunization were carried out essentially as described (4). Briefly, bovine thyroglobulin (30 mg) was dissolved in 2 mL of H_2O, and 10 mg of freshly prepared compound 1 in 0.5 mL of CH_3OH was added. After 30 min, 3 mg of sodium cyanoborohydride was added. The reduction was allowed to continue for 24 hr at 4°C. The mixture was then dialyzed against H_2O for 24 hr with the water being changed three times. The conjugate (1 mg) in 1 mL of H_2O was emulsified with an equal volume of complete Freund adjuvant and injected intradermally into the back of a female rabbit (8–10 wk old) at multiple sites. Booster injections at the same dose were carried out at monthly intervals. the rabbit was bled through an ear vein 7–14 days following each booster injection. Plasma was obtained from blood after centrifuging the blood at 2,000 × *g* for 10 min. Plasma (1 mL) was mixed with 1 mL of the binding buffer for the Affi-Gel protein A MAPS II Kit. The clear solution after filtration was applied onto a 2-mL Affi-Gel protein A column equilibrated with the binding buffer. The column was washed with 5 mL of the binding buffer which eluted plasma proteins other than immunoglobulinG (IgG). The IgG fraction was eluted with 2 mL of the elution buffer from the kit and extensively dialyzed against H_2O. The IgG fraction was used for radioimmunoassay.

Isolation of rabbit neutrophils. Rabbit neutrophils were isolated as described previously with some modifications (5). More than 98% of the neutrophils were viable as verified by trypan blue staining.

Incubation conditions and PAF extraction. Neutrophils (1×10^6) in 0.5 mL of Hanks balanced salt solution containing 0.1% bovine serum albumin were stimulated with appropriate concentrations of A23187 or FMLP at 37°C. The reaction was terminated at the time indicated by adding 1.9 mL of $CHCl_3/CH_3OH/CH_3COOH$ (1:2:0.04, v/v/v). The phases were separated by the procedure of Bligh and Dyer (6), and the lower layer was removed and evaporated under a stream of nitrogen. For PAF determinations (in duplicates), the residue was dissolved in the radioimmunoassay buffer.

Radioimmunoassay of PAF. The assay was carried out as previously described (4). The assay was run, in duplicates, in 12×75 mm conical polypropylene tubes. The standard assay buffer, 0.1 M sodium phosphate buffer pH 7.0 containing 0.15 M NaCl, 0.05% bovine serum albumin, and 0.005% Tween 20, was used for diluting antibodies, PAF standard, samples, and labeled PAF. The assay mixture contained 0.2 mL of PAF standard or sample, 0.04 mL of appropriately diluted antibodies, 0.04 mL of tritiated PAF (*ca.* 15,000 cpm), and 0.02 mL of goat anti-rabbit IgG in a final volume of 0.4 mL of the standard assay buffer. The mixture was incubated at 4°C overnight, and IgG was precipitated by adding 2 mL of cold 4% polyethylene glycol 6000. After centrifugation at 3,000 rpm for 30 min at 4°C, the supernatant was removed and the precipitate was resuspended in 1 mL of H_2O. The radioactivity of the suspension was determined by liquid scintillation counting. The concentration of PAF was estimated from standard curve.

RESULTS AND DISCUSSION

PAF does not possess functional groups that can readily be conjugated to proteins. The synthesis of a PAF analog with reactive conjugatable function is a prerequisite for the preparation of a hapten-protein conjugate. We synthesized an aldehydic PAF analog (compound 1, Scheme 1) which reacted readily with thyroglobulin. Subsequent reduction with sodium cyanoborohydride provided a stable conjugate. We have chosen a shorter alkyl chain at *sn*-1, which is structurally distinct from most phospholipids, for eliciting immune response.

Rabbits produced detectable antibodies against the PAF analog conjugate after injection over 2 mon. Binding of tritiated PAF to antibodies was not detected when crude antiplasma was diluted and used for incubation. However, binding was observed when the IgG fraction of the antiplasma or acidified antiplasma was used. The lack of binding by crude antiplasma may be due to rapid deacetylation of the labeled PAF by acetylhydrolase which has been reported to be present in plasma (7). Since the purified IgG fraction showed higher binding and less nonspecific background than acidified plasma, the IgG fraction was routinely used in the radioimmunoassay.

The radioimmunoassay of PAF described here can detect 20 pg of PAF per assay tube at a final antibody dilution for 1 to 1,000 (Fig. 1). The concentration of PAF which inhibits 50% maximal binding (IC_{50}) is 300 pg, as shown in Figure 1 and Table 1. Racemic PAF gave an IC_{50} of 600 pg, *i.e.* twice as much as IC_{50} of PAF indicating stereospecific binding of the antibodies. The specificity of the antibodies was further examined by measuring competitive binding of various phospholipids

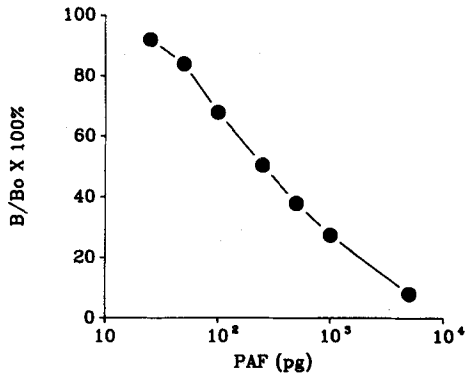


FIG. 1. Standard displacement curve for PAF. The radioimmunoassay was carried out as described in Materials and Methods. B_0 represents % binding of labeled PAF in the absence of unlabeled PAF. B represents % binding of labeled PAF in the presence of the indicated amount of unlabeled PAF.

TABLE 1

Crossreaction of Anti-PAF Antibodies with Phospholipids[a]

Phospholipids	IC_{50}	% Crossreaction
PAF	300 pg	100
rac-PAF	600 pg	50
LysoPAF	>50 μg	<0.02
PC	>25 μg	<0.04
LysoPC	>50 μg	<0.02
PI	>50 μg	<0.02
LysoPI	>15 μg	<0.06
CPL		ND
PE		ND
LysoPE		ND
PS		ND
LysoPS		ND

[a]ND, not detectable; CPL, choline plasmalogen; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; lysoPAF, lysoplatelet-activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine.

including lysoPAF and plasmalogens with labeled PAF. Only insignificant crossreaction with lysoPAF and the other phospholipids was observed, indicating that the antibodies recognize the acetyl moiety of PAF. PAF was also found to be released to an insignificant extent into extracellular space (8). Therefore, quantitation of the total amount of PAF synthesized requires lipid extraction from the cells. The Bligh and Dyer method (6) was shown to effectively extract PAF and was therefore used in the present study. Since the antibodies did not appear to cross react with any of the phospholipids other than PAF, the crude extract was dissolved in the buffer and directly used for the radioimmunoassay. The validity of the assay was ascertained by measuring PAF in increasing volumes of the extract and by examining whether the PAF levels measured increased in a linear manner. A linear correlation was observed. Furthermore, when known amounts of PAF were added to an extract, the recovery of added PAF as measured by radioimmunoassay (RIA) was essentially quantitative ($87 \pm 8\%$; $n = 7$).

When rabbit neutrophils were stimulated with 1 μM

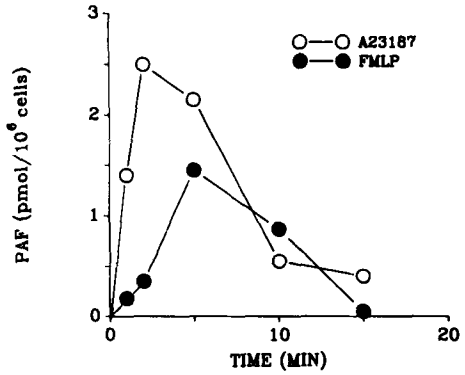


FIG. 2. Time course of PAF synthesis in rabbit neutrophils following stimulation by A23187 or FMLP. Rabbit neutrophils (2×10^6/mL) were stimulated by 1 μM A23187 or by 0.1 μM FMLP for the indicated length of time. Extraction and radioimmunoassay of PAF were carried out as described in Materials and Methods.

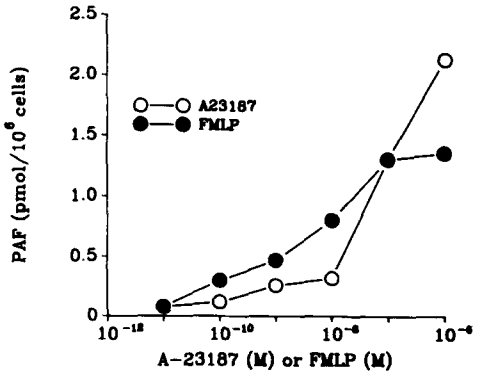


FIG. 3. Concentration dependent PAF synthesis in rabbit neutrophils following stimulation by increasing concentrations of A23187 or FMLP. Rabbit neutrophils (2×10^6/mL) were stimulated by the indicated concentrations of A23187 for 2 min or of FMLP for 5 min. Extraction and radioimmunoassay of PAF were carried out as described in Materials and Methods.

calcium ionophore A23187, rapid synthesis and accumulation of PAF was observed peaking at 2 min as shown in Figure 2. The level of PAF decreased to near basal after 10 min of stimulation. Similarly, 0.1 μM FMLP stimulated rabbit neutrophils to synthesize PAF with a peak at 5 min. Again the level of PAF returned to near basal 15 min after stimulation. Stimulation of rabbit neutrophils by A23187 or FMLP was found to be concentration-dependent as shown in Figure 3. A significant increase in PAF synthesis could be observed with 0.1 nM FMLP or 1 nM A23187, and near maximal synthesis was achieved at 1 μM FMLP or 10 μM A23187. A23187 appeared to be the more potent agonist for inducing PAF synthesis. The peak levels of PAF were in the range of 1.5–2.5 pmol/10^6 cells. The figures are similar to those reported for human neutrophils stimulated with FMLP (9) and for rat hepatic Kupffer cells following A23187 stimulation (10), but were significantly lower than those reported for human endothelial cells stimulated with thrombin (8).

Antibodies against PAF have been described in some previous reports. In one study, rabbits were immunized with liposomes containing PAF. However, the antibodies showed poor specificity and affinity for PAF (11). In another study, an acetylhydrolase resistant analog, an *N,N*-dimethylcarbamoyl derivative of ω-carboxylated PAF was synthesized and conjugated to bovine serum albumin (12). Although the conjugate elicited antibody production, the antibodies showed some crossreaction with lysoPAF. An approach similar to ours of synthesizing an aldehydic analog of PAF for antibody production was published during the course of our study (13). The authors employed a different synthetic route to synthesize dimethoxy PAF analogs which were then converted to the corresponding aldehydes by acid hydrolysis. The antibodies showed good specificity, but lower sensitivity (2.5 ng per assay tube). The radioimmunoassay described herein represents a specific and sensitive method capable of quantitating PAF in biological samples.

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REVIEW

Metals and Lipid Oxidation. Contemporary Issues[1]

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Lipid oxidation is now recognized to be a critically important reaction in physiological and toxicological processes as well as in food products. This provides compelling reasons to understand what causes lipid oxidation in order to be able to prevent or control the reactions. Redox-active metals are major factors catalyzing lipid oxidation in biological systems. Classical mechanisms of direct electron transfer to double bonds by higher valence metals and of reduction of hydroperoxides by lower valence metals do not always account for patterns of metal catalysis of lipid oxidation in multiphasic or compartmentalized biological systems. To explain why oxidation kinetics, mechanisms, and products in molecular environments which are both chemically and physically complex often do not follow classical patterns predicted by model system studies, increased consideration must be given to five contemporary issues regarding metal catalysis of lipid oxidation: hypervalent non-heme iron or iron-oxygen complexes, heme catalysis mechanism(s), compartmentalization of reactions and lipid phase reactions of metals, effects of metals on product mixes, and factors affecting the mode of metal catalytic action.
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Metal catalysis of lipid oxidation has been recognized for decades. Early research focussed on oxidation of food lipids, and it was in this area that there was intense research effort during the 1960s. Most attention at that time was given to determining lipid oxidation kinetics and catalytic mechanisms of the metals, including which metals, which chelates or complexes, and which valence states were most active (1-11). During the early 1970s, the feeling that the research of the 1960s had discovered all the answers led to metal catalysis of lipid oxidation being considered, for the most part, passé. However, during the late 1970s and early 1980s, the realization that metals play an important, if not critical role in oxidative cytotoxicity reawakened interest in metal catalysis (12-19). Oxidative cytotoxicity refers to pathological processes presumed to be caused by reduced forms of oxygen including H_2O_2, $O_2^{\cdot-}/HO_2^{\cdot}$ (may react directly or dismutate to H_2O_2), $HO^{\cdot}$ from reduction of H_2O_2, and lipid alkoxyl or peroxyl radicals. Production of these radicals is driven catalytically by the trace levels of iron, copper, and perhaps other redox-active metals present in tissues (14,15,18,20,21). Indeed, many scientists feel that participation of metals is *obligatory* for toxic fluxes of radicals which may overwhelm natural defense mechanisms in some diseases and in chemical and drug toxicities (18,20,22,23).

Largely due to the interest of the toxicologists, research on metal catalysis of lipid oxidation has been renewed and revitalized during the 1980s. Food scientists, too, are finding new problems with metals relative to the stability of lipids, particularly in multiphasic foods. Several contemporary issues in metal catalysis have evolved from attempts to understand and explain why oxidation kinetics, mechanisms, and products in molecular environments which are both chemically and physically complex (as in foods and tissues) often do not follow the classical patterns predicted from model system studies.

The general chemical mechanisms for metal catalysis of lipid oxidation are quite familiar (3,24-27).

DIRECT INITIATION

1) by higher valence state metals, via electron transfer and formation of lipid alkyl radicals (2,24). Iron and copper (28) are known to behave this way, as do Mn, Ni, and Co (29,30). For LH representing an unsaturated lipid molecule

$$M^{(n+1)+} + LH \rightarrow M^{n+} + L^{\cdot} + H^{+} \quad [1]$$

2) by lower valence state metals. a) *via* formation of metal-oxygen transition complexes ($M^{(n+1)+} \ldots O_2$), particularly in non-polar solvents (2,3)

$$M^{n+} + O_2 \rightarrow (M^{(n+1)+} \ldots O_2) \rightarrow \begin{cases} M^{(n+1)+} + O_2^{\cdot-} & [2a] \\ \xrightarrow{LH} L^{\cdot} + (M^{n+1)+} \ldots {}^{-}O_2H) & [2b] \\ \xrightarrow{LH} L^{\cdot} + M^{n+} + HO_2^{\cdot} & [2c] \\ \xrightarrow{LH} LO^{\cdot} + (M^{(n+1)+} \ldots OH^{-}) & [2d] \\ \xrightarrow{(M^{n+}LH)} HO_2^{\cdot} + (M^{(n+1)+}L^{-}) + M^{n+} & [2e] \end{cases}$$

b) *via* metal autoxidation, which produces reactive oxygen species, $O_2^{\cdot-}/HO_2^{\cdot}$ and H_2O_2

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{\cdot-} \xrightarrow{H^{+}} HO_2^{\cdot} \quad [3]$$

$$O_2^{\cdot-}/HO_2^{\cdot} \rightarrow H_2O_2 + O_2 \quad [4]$$

Reaction of $O_2^{\cdot-}$ with double bonds of unsaturated fatty acids was proposed as early as 1962 (4), but pulse radiolysis studies have shown that it is $HO_2^{\cdot}$ rather than $O_2^{\cdot-}$ that reacts with unsaturated fatty acids (31,32), and

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Abbreviations: ADP, adenosine diphosphate; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; NHE, normal hydrogen electrode; NMR, nuclear magnetic resonance; SOD, superoxide dismutase; TBA, thiobarbituric acid.

the reaction is slow ($k = 300 - 1700\ M^{-1}\ sec^{-1}$) (31,33, 34). Reaction of $O_2^{\cdot}$ /$HO_2^{\cdot -}$ with lipid hydroperoxides has been reported (35), but a later study showed that scrupulously demetalled LOOH is not decomposed by $O_2^{\cdot -}$ (36).

More important is the reduction of H_2O_2 to yield hydroxyl radicals, HO$^{\cdot}$ (37), which initiate lipid oxidation by hydrogen abstraction ($k = 8 \times 10^9\ M^{-1}s^{-1}$ for linolenate) (32,38)

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + HO^{\cdot} + OH^- \quad [5]$$

$$HO^{\cdot} + LH \rightarrow L^{\cdot} + H_2O \quad [6]$$

INDIRECT INITIATION, PROPAGATION, OR CHAIN BRANCHING

Catalysis by some metals (Fe, Cu, Ni) occurs by oxidation (39,40) or reduction (41) of preformed lipid hydroperoxides (LOOH) to form chain-carrying LO$^{\cdot}$ and LOO$^{\cdot}$ radicals. Using iron as an example

$$LOOH + Fe^{3+} \rightarrow LOO^{\cdot} + H^+ + Fe^{2+} \quad [7]$$

$$LOOH + Fe^{2+} \rightarrow LO^{\cdot} + OH^- + Fe^{3+} \quad [8]$$

According to traditional theory, these hydroperoxide decompositions increase the rate of chain re-initiation or propagation because the rates of hydrogen abstraction by LO$^{\cdot}$ and LOO$^{\cdot}$ are much faster than the rates of *ab initio* L$^{\cdot}$ formation (24). Recent evidence, however, has suggested that secondary epoxyallylic radicals from LO$^{\cdot}$ rearrangements may be more likely chain carriers under some conditions (42).

The redox potentials of other metals such as Mn and Co are too low to effect LOOH decomposition in aqueous systems (41), but they may catalyze hydroperoxide decomposition in non-polar media by formation of metal-hydroperoxide complexes (1,40,43).

$$Co^{3+} + LOOH \rightarrow (LOOHCo)^{3+} \rightarrow Co^{2+} + LO_2^{\cdot} + H^+ \quad [9]$$

$$Co^{2+} + LOOH \rightarrow (LOOHCo)^{2+} \rightarrow Co^{3+} + LO^{\cdot} + OH^- \quad [10]$$

Inhibitory effects of metals are also known, usually when metals are present at higher concentrations (10,25, 43–46). The inhibition is thought to result from oxidation and reduction of free radicals by iron and copper (24,37, 47,48) and from metal complexation of free radicals by Co (43,49). Either mechanism results in interruption of the free radical chain and reduction in the overall oxidation rate.

$$\text{(Fe, Cu)} \quad L^{\cdot} + M^{(n+1)+} \rightarrow (L^{\cdot} \ldots M^{(n+1)+}) \text{ (stable)} \quad [11a]$$

$$\text{(Fe, Cu)} \quad L^{\cdot} + M^{(n+1)+} \rightarrow M^{n+} + L^+ \rightarrow \text{products} \quad [11b]$$

$$L^{\cdot} + M^{n+} \rightarrow M^{(n+1)+} + L^- \quad [12]$$

$$\text{(Co, Mn)} \quad R^{\cdot} + MA_n \rightarrow R\text{-}MA \quad [13]$$

where R$^{\cdot}$ may be L$^{\cdot}$, LO$^{\cdot}$, LOO$^{\cdot}$, etc.

These mechanisms have been derived from studies conducted in relatively simple chemical model systems of defined composition with controlled reaction conditions. Biological systems, on the other hand, are more complicated. They have multiple reaction environments in membranes and aqueous phases, and natural materials have complex compositions which are difficult to reproduce and control precisely. Metal catalysis of lipid oxidation in these complex biological systems is not as uncomplicated as classical mechanisms would suggest.

For example, both valence forms of metals have catalytic activity, and the factors affecting the balance or dominance between direct initiation reactions and re-initiation by hydroperoxide decompositions are poorly understood. Also, problems in the interpretation of mechanisms in complex biological systems have arisen when the reaction kinetics or the products did not obviously fit these classical mechanisms. This has been particularly true for toxicological studies which tried to determine whether HO$^{\cdot}$ produced from H_2O_2 in Fenton reactions (37,47) initiate oxidation of membrane lipids.

Clearly, new understandings of metal catalysis of lipid oxidation in biological systems must be sought. Issues needing clarification are, by their nature, complex and interdisciplinary, and solutions will require input from many different fields: metallochemistry, electrochemistry, organic chemistry, physical chemistry, biochemistry, physiology and toxicology, food chemistry. To provide a context and to stimulate renewed consideration of mechanisms along with new research approaches, the following overview of contemporary issues in metal catalysis of lipid oxidation is offered. A summary of these issues is presented in Table 1. While the discussion will focus on iron because it is the dominant redox-active metal in biological systems, most of the issues are relevant also to other redox-active metals.

HYPERVALENT IRON OR IRON-OXYGEN COMPLEXES

One of the major, and perhaps one of the most controversial, contemporary issues in regard to mechanisms of lipid oxidation is whether hypervalent iron (iron valence of +4 to +6, Table 2) or other iron-oxygen complexes form and are catalytically active in biological systems in the absence of a protein or other macromolecular prosthetic group. If they do form, what are the necessary conditions, and how do the kinetics and products of their reactions compare with those produced in classical Fe^{3+}/Fe^{2+} reactions?

Originally proposed by Hochstein *et al.* (50), the principal proponents of this theory applied to biological systems have been proposed by Aust and colleagues (51–55). Based on repeated observations that maximum lipid oxidation occurs when Fe^{2+} and Fe^{3+} are present in a 1:1 ratio, they proposed a mechanism in which the species actively catalyzing lipid oxidation in the presence of H_2O_2 is ferryl iron or mixed metal-O_2 complexes [Fe^{2+}-O_2-Fe^{3+}] rather than HO$^{\cdot}$. Recently, these authors have modified their proposed mechanism, contending that 1:1 ratios of ferric and ferrous iron facilitate redox cycling of the iron. Details of this theory and a review of their work are presented in this issue (56).

Authentic hypervalent iron complexes have long been recognized as active forms of heme proteins and porphyrin

TABLE 1

Summary of Contemporary Issues Which Need to Be Clarified Regarding Metal Catalysis of Lipid Oxidation

1. HYPERVALENT NON-HEME IRON OR IRON-OXYGEN COMPLEXES:
   - Under what conditions do they form in biological systems?
   - Do they catalyze lipid oxidation?
   - What are the characteristic kinetics and lipid products?
2. HEME CATALYSIS MECHANISM(s):
   - Hydroperoxide reduction, oxidation by hypervalent iron, or other mechanisms?
3. COMPARTMENTALIZATION OF REACTIONS AND LIPID PHASE REACTIONS OF METALS:
   - Where does metal catalysis occur in multiphase systems?
   - Are lipid phase reactions of metals significant?
   - Do catalysis mechanisms and oxidation products differ depending on phase?
4. EFFECTS OF METALS ON PRODUCT MIXES:
   - Does metal catalysis influence isomeric hydroperoxide distributions, alkoxyl radical scission reactions, intramolecular rearrangements to epoxides, etc.?
5. FACTORS AFFECTING THE MODE OF METAL CATALYTIC ACTION—direct initiation *vs* chain propagation (reinitiation), mechanism, reaction location, overall course of oxidation:
   - Chelator—partition coefficients, redox potentials, pK_a.
   - Reaction conditions—metal concentrations, pH, presence/absence of water and reducing agents, oxygen tension, type of lipid.
   - Membrane surface charge.

TABLE 2

Formulas and Nomenclatures for Hypervalent Iron Complexes

$(FeO)^{2+}$	$(FeO)^{3+}$	$(FeO_4)^{2-}$
Fe(IV)	Fe(V)	Fe(VI)
Ferryl	Perferryl	Ferrate
MIXED METAL-OXYGEN COMPLEXES:		
Fe^{2+}-O_2-Fe^{3+}		

compounds. Hypervalent iron states also have been identified and characterized spectroscopically in numerous enzymes and in iron complexes in organic solvents, but little is known about hypervalent iron in aqueous solutions. Physical chemists have expressed great skepticism that hypervalent iron occurs in non-heme small molecular complexes under physiological conditions on the grounds that hypervalent iron complexes require *macro*molecular complexers or a very high pH for formation and stabilization. Thus, hypervalent iron should not be a significant reactant since in complex biological systems decay of hypervalent states to Fe^{3+} would be essentially instantaneous, eliminating any enhanced catalytic capability.

There have been a few reports based on electron paramagnetic resonance (EPR) (57,58) and kinetic spectroscopic (59) evidence for the formation of hypervalent iron in small molecular complexes. However, the hypervalent iron indeed decayed very rapidly to its ferric form. In other reports, hypervalent iron was thought to be formed in olefin microemulsions, but only at high (10:1) hydroperoxide/iron ratios (60); 1:1 ratios yielded conventional Fenton chemistry.

Bielski (61) studied the stability and reactivity of synthetic Fe^{4+}, Fe^{5+}, and Fe^{6+} complexes in aqueous solutions, and found that all the complexes were strong oxidants at high pH. Fe^{+4} and Fe^{+5} were significantly more reactive than Fe^{+6}, due to substantial free radical character of the Fe–O bonds in the ferryl/perferryl species. Proton sources in solvent or substrate markedly increased decay rates of the hypervalent states.

While Bielski's results suggest that hypervalent iron states, *if* they form in contact with lipids in biological systems, should be particularly potent initiators of lipid oxidation, three critical issues must still be addressed to understand the practical importance of these complexes: i) Under what conditions do these hypervalent states form in small molecules under physiological conditions? What is the source of oxygen, is it dissolved molecular oxygen or hydrogen/hydroperoxides? What pH? What complexing agents? If aprotic solvents stabilize hypervalent states, may the hydrophobic lipid phases of membranes be preferential sites for hypervalent iron formation and activity? ii) Do hypervalent iron complexes actually catalyze lipid oxidation? Under what conditions? What are the rate constants? iii) How do the kinetics, mechanisms, and products differ from normal autoxidation or Fe^{2+}/Fe^{3+} catalyses? Are hypervalent iron processes distinguishable from other oxidations?

HEME CATALYSIS MECHANISM(S)

Early observations that heme compounds were catalytically active in the ferrous form and that no valence change of the iron occurred in the process led Tappel (5,62) to propose a mechanism by which lipid hydroperoxides form activated complexes through a coordinate bond between the ferrous heme and the hydroperoxide function. At the time, no distinction could be made between radical scission of the hydroperoxide,

$$\text{LOOH} + \text{Heme-Fe}^{2+} \rightarrow \text{Heme-Fe}^{2+} + \text{LO}^{\bullet} + {}^{\bullet}\text{OH} \quad [14]$$

and reductive scission followed by secondary oxidation of ions to immediately recycle the ferric to ferrous heme (5).

$$\text{LOOH} + \text{Heme-Fe}^{2+} \rightarrow \text{Hematin-Fe}^{3+} + \text{LO}^{\bullet} + \text{OH}^{-} \quad [15a]$$

$$\text{or Hematin-Fe}^{3+} + \text{LO}^{-} + {}^{\bullet}\text{OH} \quad [15b]$$

$$\text{Hematin-Fe}^{3+} + \text{OH}^{-} \rightarrow \text{Heme-Fe}^{2+} + {}^{\bullet}\text{OH} \quad [16a]$$

$$+ \text{LO}^{-} \rightarrow \text{Heme-Fe}^{2+} + \text{LO}^{\bullet} \quad [16b]$$

Tappel's mechanism is historically important, as it explained most observations for many years. Recent

advances in analytical techniques have enabled others to provide amplifications and mechanistic details to Tappel's theory. Bruice (63) has presented evidence that in aqueous solution radical scission dominates with the alkoxyl radical being released and the ·OH remaining bound to the heme compound, while in organic solvents radical scission occurs followed by secondary electron transfer such that products are equivalent to those which would be produced by reductive O–O bond cleavage. However, not all hemes follow this mechanism, so a diagnostic probe to distinguish which heme compounds decompose hydroperoxides by mechanisms yielding alkoxyl radicals has been developed using 10-hydroperoxy linoleic acid (64,65).

However, reductive scission of hydroperoxides alone does not adequately explain differences between catalytic efficiencies or the specific product mixes of different hemes (26,66). Also, recent EPR studies have shown clearly that ferric as well as ferrous hemes are active catalysts, yielding peroxyl radicals from hydroperoxides (67,68). Inconsistencies in pro-oxidant effects of hemes continue to be reported, probably because multiple mechanisms of heme catalysis exist, even for individual hemes, determined by reaction conditions and components and by the specific heme compounds involved.

Contemporary studies have provided evidence for at least four mechanisms by which heme compounds may catalyze lipid oxidation in addition to the straightforward electron transfers described in Reactions 7 and 8.

One mechanism is the formation of hypervalent iron complexes which oxidize lipids directly. In the discussion about hypervalent iron above, the qualifications "small molecule" or "non-heme" were repeatedly used because it is known clearly that ferryl iron is formed by peroxidase (69) and other heme proteins and is the active oxidizing form of these enzymes (70,71). Ferryl complexes are also formed in catalase (69,72), cytochrome P-450 (73), myoglobin (74,75), hemoglobin (57,58,76), and model porphyrins (63,77–79), although less is known about the catalytic activity of ferryl forms of these proteins.

All of these heme proteins have been shown to catalyze lipid oxidation, and by analogy to the above it seems logical to question whether hypervalent iron may be involved in this activity, formed *via* either heme-oxygen, heme-hydrogen peroxide, or heme-lipid hydroperoxide complexes. The requirement for at least one of these oxygen sources in order for catalysis of lipid oxidation to occur has been noted in tissue (80) and chemical model reaction systems (81). Observations that metmyoglobin catalysis in air was markedly accelerated as pH was increased, especially above pH 7 (81), is consistent with this hypothesis since hypervalent iron complexes are more stable at high pH (61). Hypervalent iron activity may also explain observations that hemoglobin catalyzes oxidation of linoleic acid and ester (82) and phospholipids in liposomes (83) without induction periods, and that haptoglobin inhibits hemoglobin-stimulated lipid oxidation (84). Reduction of hypervalent iron or prevention of its formation may contribute to antioxidant effects of high heme concentration (85) and the reducing agents ascorbic acid and cysteine (85).

A second mode of heme catalysis may involve an indirect mechanism in which HO· attacks lipids to initiate autoxidation chain reactions. The HO· arises from either a) the autoxidation of ferrous heme iron to produce $O_2^{\cdot-}$ and H_2O_2, which is then reduced to HO· by adventitious iron (86,87); or b) heme-catalyzed reduction of H_2O_2 added or produced *in situ* (88).

A third possible mechanism for catalysis of lipid oxidation by hemes is photosensitization, either *via* free radicals or singlet oxygen (89). Ten years ago this concept was proposed on theoretical grounds (26) and recently some experimental verification for Type I (free radical) sensitization has been published (90).

Recent observations suggest that a fourth mode of heme catalysis is also possible in systems containing H_2O_2: HO· attacks hemoglobin (or other heme proteins) and releases iron which then catalyzes lipid oxidation as non-heme iron (91,92).

Of these mechanisms, hypervalent iron states seem to account best for many characteristics of heme catalysis of lipid oxidation documented but incompletely explained in the previous literature. Nevertheless, several issues still require clarification, including which valence states of hemes are involved, whether different hemes catalyze lipid oxidation by different mechanisms, what conditions favor activity of hypervalent iron as opposed to direct reduction of hydroperoxides or any of the other mechanisms which have been proposed, and which molecular regions are involved in the electron transfers, *i.e.* whether the iron atom, porphyrin ring, or apoprotein (93) is involved.

COMPARTMENTALIZATION OF REACTIONS AND LIPID PHASE REACTIONS OF METALS

Apparent inconsistent effects of iron on oxygen radical reactions and lipid oxidation in biological systems have been observed, but this may be expected since cells, tissues, membranes, and organelles, by their nature, lack the precisely controlled conditions and defined composition of laboratory test tube reactions. For example, adenosine diphosphate (ADP), ethylenediaminetetraacetic acid (EDTA), and histidine complexation of iron may have pro- or antioxidant activity or no effect on lipid oxidation depending on the source and preparation of tissues, cells, membranes or purified lipid, the solvents used, the concentrations of metal complexes, the presence or absence of oxygen sources, and how the effects are measured. Because we need to be able to predict and control metal reactions in foods and in living tissue, we have cogent and compelling reasons to understand the molecular bases for these inconsistencies and thus to determine the physical and chemical factors which affect iron activity in complex, multiphase, and often compartmentalized systems.

One critical physical aspect which cannot be ignored is that biological systems are always at least *bi*phasic, inhomogeneous systems, *i.e.* a hydrophilic, protic aqueous phase and a hydrophobic lipid phase which may have both protic and aprotic regions. Because of the bilayer structure of the membrane, initiation of lipid oxidation must necessarily occur in the membrane interior region, where the lipid chains are located. However, in typical experimental designs, metal reagents are added to an external aqueous phase. Reactions occurring in the aqueous phase can initiate lipid oxidation only if reactive intermediates or products diffuse to the surface or into the lipid bilayer of the membrane. Because of their high reactivity, HO· generated in the aqueous phase have little, if any, chance of initiating oxidation of lipids in membranes or in lipid

phases in food products. Water-soluble chelators or free radical interceptors such as mannitol, superoxide dismutase (SOD), and catalase can be effective against reactants in the aqueous phase, but are unlikely to affect oxidations occurring in the hydrophobic regions of lipid bilayers.

This paradox highlights a third important issue regarding metals and lipid oxidation: Where does metal catalysis of lipid oxidation occur in a multiphase system? What reactions of metals occur in lipid phases and how do their mechanisms, kinetics, and products differ from aqueous phase or interface reactions of metals?

Traditional chemistry holds that catalysis in a biphasic system will occur at the phase interface or at the membrane surface. Thus, any chelators or reaction conditions which increase the binding of metals to the membrane, creating "ill-placed" metal (94), should increase the catalytic effectiveness of the metal. It is well-known, for example, that copper binds readily to membrane proteins and mediates site-specific reactions at those locations (22,95,96). Iron also has surface binding sites on proteins (97,98) and binds to the phosphoric acid moieties of phospholipids (99–101). This binding contributes to rapid iron uptake by membranes, and to reduction of lipid hydroperoxides near those sites. Zinc (102–104) and other metals can displace iron from these sites, but whether aqueous phase $HO^{\cdot}$ interceptors and metal chelators have access to and can compete with binding sites on the membrane surface remains to be determined.

Considering that lipid molecules themselves are the final targets and participants in lipid oxidation, surprisingly little attention has been given to the reactions which may occur in the hydrophobic lipid region of the membrane (or lipid phases of foods), catalyzed by metal complexes, H_2O_2, reducing agents, or other reactants which diffuse or solubilize there. This may be partially attributed to a general expectation that metals are not soluble in hydrophobic media. Nevertheless, fatty acid complexation of metals has long been known to occur and is responsible for a major proportion of metal contamination of refined food oils. In recent studies determining lipid partitioning into lipid phases, fatty acids solubilized metals at concentrations of micromolar or higher, depending on the chelating or complexing agent; complexation of metals through the carboxylic acid groups was very likely involved (105). ADP and (des)ferrioxamine were surprisingly potent solubilizers, a characteristic which may partially explain why ADP as a chelator is unusually effective in enhancing lipid oxidation in membranes, and why desferrioxamine is not always protective against oxidative damage in tissues.

Iron complexes were 10-100× less soluble in the aprotic methyl esters of fatty acids, but even 10^{-8} M concentrations of iron were sufficient to drive oxidations detectable within minutes (seconds in some cases) by EPR spin trapping in these pure lipid phases (105).

Why should lipid phase reactions be critical? Reactions of metals occurring in the lipid phase, whether direct initiation, Fenton generation of $HO^{\cdot}$, or decomposition of LOOH, would be inaccessible to water-soluble interceptors. Thus, negative results in experiments using inhibition of lipid oxidation by water-soluble agents to derive mechanisms can be misinterpreted; rather than the causal connection being absent, the causal location may be wrong.

Perhaps an even more important reason is that Fenton and other iron reactions in aprotic solvents such as acetonitrile or lipid esters may not proceed as they would in water or protic organic solvents. Several factors may contribute to these reaction differences. First, the electrochemistry of iron (196) and the reactions of product oxyl radicals ($O_2^{\cdot-}/HO_2^{\cdot}$, $HO^{\cdot}$, $RO^{\cdot}$, and $ROO^{\cdot}$) in aprotic organic solvents differ from their aqueous counterparts. Ferric oxidation of hydroperoxides, which are relatively weak in aqueous solutions, are accelerated in aprotic solvents such as acetonitrile (107,108). Changes in the ligand structure and solvation state of the metal may shift electron transfer from an outer sphere (free radical) to an inner sphere (peroxide complex) process (39), thus altering oxidation mechanisms and products (108). Fe^{2+} has long been known to autoxidize much more rapidly (109), and hypervalent iron states form more readily and are more stable in aprotic solvents. Recent evidence that ferric iron is not easily reduced in aprotic fatty acid methyl esters (Schaich, K.M., unpublished data) is consistent with observed iron behavior in other aprotic organic solvents (110).

These observations may be extrapolated to membranes. Because the acyl chains forming the hydrophobic region of the lipid bilayer should be aprotic while the phosphate regions near the surface should be protic, the mechanisms of metal catalysis and the processes of lipid oxidation in these two regions are very likely to be different.

In research with Fenton and metal reactions in lipid phases, reactions in protic fatty acids have been shown to clearly differ from those in aprotic esters (105). EPR spin trapping studies of Fenton reactions (Fe^{3+}, H_2O_2, and hydroxylamine as a reducing agent) in lipid phases showed rapid Fenton reactions generating $HO^{\cdot}$ which could be trapped in fatty acids, but in fatty acid esters Fenton reactions were slower and $HO^{\cdot}$ were never trapped; instead, complex multiple-species spectra were produced.

Initial EPR spectra in lipid esters showed mixtures of radicals (105). Because Fe^{3+} reduction was inhibited and its oxidations were accelerated in the lipid phases, three oxidation reactions competed to produce the initial radicals: i) oxidation of H_2O_2 to $HO_2^{\cdot}$; ii) direct oxidation of unsaturated lipids to yield lipid peroxyl radicals (oxygen was present); and iii) oxidation of traces of preformed lipid hydroperoxides to peroxyl radicals. At later reaction stages, an unusual spectrum appeared; this was assigned to an epoxyallylic radical species.

Additional evidence that metal reactions in lipid or other non-aqueous aprotic phases do not follow classical aqueous reaction pathways has been provided in new reports showing production of ketones at the alkoxyl radical carbon (111,112) or a variety of epoxide or other rearrangement products rather than scission products when lipid hydroperoxides are reduced by iron complexes (113) or by ultraviolet light (114) in aprotic solvents. Similarly, in a reaction system containing $FeCl_3$, H_2O_2, and olefins in acetonitrile, epoxide and dioxetane products were formed rather than peroxy radicals, hydroxylation or scission products (115). An Fe^{III}-oxene [$Fe^{III}(O)$] species was proposed as the active catalyst in the latter study.

Another possible explanation for differences in oxidation mechanism in different media is offered by observations that linoleic acid and linoleate-containing phospholipids autoxidized neat, in chlorobenzene, or in buffered

vesicular systems yield hydroperoxides with very different *cis,trans* to *trans,trans* ratios (116–118). The *cis,trans/trans,trans* isomer ratios decreased as lipid concentrations increased, as hydrogen donating ability of the solvent decreased, and as intermolecular lipid associations increased. Analogous hydroperoxide patterns have been observed following *in vivo* lipid peroxidation (119). Thus, molecular conformations of hydroperoxides in lipid phases may facilitate isomerization and intramolecular rearrangements over scission reactions. A third factor which may favor intramolecular rearrangement is a reduction in the rate of intermolecular hydrogen abstraction by LOO$^{\bullet}$ due to the viscosity of lipid phases (120).

Additional research will be needed to determine definitively which effects of solvent have the dominant influence on metal catalyses of lipid oxidation: alteration of the electrochemistry of iron complexes, proton transfer mediation, changes in lipid conformations and molecular associations, or still other factors as yet unrecognized.

EFFECTS OF METALS ON PRODUCT MIXES

Numerous studies have documented the classical breakdown of oxidizing lipids into mixtures of hydroperoxides, aldehydes, ketones and other carbonyl compounds, alkanes and carboxylic acids *via* scission reactions of the lipid alkoxyl radicals (121,122, and references therein). Less attention has been given to internal rearrangements of hydroperoxides to epoxides and related product (113, 114,123,124), and very little research has focussed on determining what factors influence scission reactions and whether metal catalysis alters reaction pathways and product distribution. How metals and metal-solvent interactions affect the overall course of lipid oxidation and the scission reactions which produce malonaldehyde and similar reactive aldehydes, and how metals influence the dominance of scission reactions yielding carbonyls *versus* rearrangement reactions yielding epoxides in different environments are specific issues which have received little attention.

Why is this important? Metals affect not only the rate of initiation and total extent of lipid oxidation, but also the degree of chain branching and secondary reaction, and the nature of termination reactions, *i.e.* metals determine which final products are formed.

The thiobarbituric acid (TBA) test is a measure of lipid oxidation commonly used as a measure of the effectiveness of metals in catalyzing lipid oxidation. This test provides very convenient analyses because the products are water-soluble, eliminating the need for tedious lipid extractions. However, the TBA test depends on the production of "expected" scission products, predominantly malonaldehyde, although cyclic peroxides and dihydroperoxides of 18:3 and higher fatty acids are also known to react (125,126). Malonaldehyde is only produced in secondary scissions of fatty acids with three or more double bonds, and it is a relatively minor breakdown product. Media or reactants may alter the oxidation pathways, increasing or even eliminating cyclization reactions and malonaldehyde production.

For example, in the presence of water, metals increase both the breakdown of LOOH products to alkoxyl radicals and α- and β-scissions of alkoxyl radicals to aldehydes, other carbonyl compounds, and alkanes. Indeed, some versions of the TBA test include an iron reagent to accelerate these scission reactions and the subsequent formation of malonaldehyde from a secondary scission reaction.

In contrast, dominant products of metal-catalyzed oxidations in neat lipid phases or in aprotic solvents appear to be rearrangement products rather than scission products (113). As discussed above, whether this results predominantly from different catalytic species (*e.g.*, iron^{III}-oxenes or hypervalent iron) or rather from direct solvent influences on proton transfers (114) and molecular associations or conformations remains to be determined.

Internal rearrangements to epoxides, hydroxyepoxides, and hydroperoxyepoxides are facilitated by close alignment and association of the lipid chains, such as would be found in oriented monolayers in dry foods and in the bilayers of membranes (127,128). Solvent also plays a critical role. As discussed above, reactions of metals in lipid phases or in lipids dissolved in aprotic solvents do not produce classical distributions of lipid scission products. The epoxide and other rearrangement products observed with the cited metal-lipid reactions are consistent with reports that olefin hydroperoxide-metal complexes yield scission products in coordinating solvents but shift increasingly to epoxide production as the hydrogen donating power of the solvent decreases (129) and the epoxides are stabilized (124).

Also, heme catalyses do not yield classical distributions of hydroxy, aldehyde, and alkane products. Multiple reaction mechanisms are recognized for many heme compounds (130) producing keto, epoxyhydroxy, trihydroxy, and other non-scission compounds as dominant products.

The practical implications of these differences are enormous for both food chemistry and toxicology. A few obvious ones can be mentioned. First, given the considerations outlined above, serious questions must be raised regarding the validity of using malonaldehyde production as an indicator of metal catalysis in a lipid phase or in compartmentalized multiphase systems such as cells. Another implication: the secondary carbonyl and alkane products resulting from scission reactions are important sources of flavors and odors, some characteristic and most undesirable, from oxidized lipids (121). If a way could be found to block this pathway, shelf-life could be extended.

Reconsideration of mechanisms by which membrane lipid oxidation is involved in biological damage processes also seems to be warranted. Lipid free radical species (20, 131–138), hydroperoxides (139–141), and carbonyl oxidation products (135,136,142–147) have commonly been blamed for co-oxidation and crosslinking of critical cellular macromolecules (136) such as tocopherols (143,148), proteins, nucleic acids (149–153), and carcinogens (154,155). Nonetheless, many lipid rearrangement products, particularly the hydroperoxyepoxides, also have been found to be cytotoxic and perhaps carcinogenic. Whether the rearrangement products are also capable of crosslinking remains to be determined, but in light of Gardner's identification of methyl linoleate epoxy adducts with cysteine catalyzed by $FeCl_3$ (124,156,157) crosslinking capability would certainly be expected. Since lipid rearrangement products would be expected to form in membrane bilayers, where proteins and nucleic acids are also bound, epoxy-mediated lipid complexation to these critical macromolecules may provide an important mechanism for the cytotoxicity and cancer promoting activity of oxidized lipids.

FACTORS AFFECTING METAL CATALYSIS

In my own studies of metal reactions in lipid phases, oxidation kinetics have proven to be complex and dependent on a number of factors, including the specific metal complex or chelator used, the valence state of the metal, the concentration of the metal, the oxygen tension of the system, the types of lipid (protic or aprotic), and the presence of contaminating preformed hydroperoxides. These are all factors well known to affect the overall kinetics of lipid oxidation, but it is less understood how these and other system variables affect the mechanism and course of metal catalysis.

Solvents, especially water, affect metal catalysis in a variety of ways. As noted above, water and other hydrophilic solvents facilitate LO$^{\bullet}$ scission reactions in solution (158). However, it is known from research of Karel and others (8,9,159) that there are also conditions in which water inhibits LOOH decompositions. Two hypotheses previously advanced to explain these actions are i) hydration of metals, thus inhibiting electron transfers to and from the metals, and ii) hydrogen bonding between water and hydroperoxides, providing stabilization. Recent nuclear magnetic resonance (NMR) studies of water-lipid hydroperoxide-metal complexes and their effects on hydroperoxide decomposition kinetics have corroborated these mechanisms (160).

Chelators and complexing agents are critical determinants of the catalytic mode and effectiveness of metals. Chelators vary in their metal affinities, their charge and solubility in lipids, and thus how they partition between lipid and aqueous phases. They differ in the valence state of iron they stabilize, the metal coordination sites they occupy, and the type of electron transfer reactions they mediate (161). They also vary in the redox potentials of their complexes (Table 3) (162,163), and hence how they poise metals for redox cycling in the presence of cellular reducing agents. These should be obvious considerations, but they are often ignored when interpreting effects of different metal complexes, particularly in multiphase systems. The net effects of chelators may be complex and seemingly contradictory.

For example, EDTA complexation of iron removes "free" or weakly complexed iron from solution. It also lowers the Fe^{3+}/Fe^{2+} redox potential from 0.77 V to 0.12 V *vs* normal hydrogen electrode (NHE). Thus, by changing the localization of iron and by limiting the capability of Fe as an oxidizing agent, EDTA can markedly reduce *ab initio* (LH → L$^{\bullet}$) initiation of lipid oxidation. However, the lower redox potential makes EDTA-iron a better reducing agent, so EDTA-Fe^{2+} chelates reduce lipid hydroperoxides faster than uncomplexed iron. In the presence of reducing agents to recycle the iron, EDTA complexation may even result in a marked acceleration of chain propagation and branching reactions (LOOH → LO$^{\bullet}$ + $^{\bullet}$OH, and LO$^{\bullet}$ + L'H → L$^{\bullet}$ + LOH). The net effect of EDTA results from the balance between these actions in individual systems, and has led to apparently contradictory reports of EDTA effects on lipid oxidation (164).

Several other chelators deserve attention. Picolinic acid, a metabolic product of tryptophan, is a strong metal chelator and is potentially very important as a physiological chelator. Picolinates have received attention recently since the iron chelates are very effective at producing HO$^{\bullet}$, at least at low H_2O_2/chelate ratios (1:1) (165). At higher hydroperoxide ratios, a mechanism more like that with hypervalent iron dominates (111,112). With such behavior, it is imperative that we understand the redox chemistry of this molecule and its chelates. Reaction characteristics of picolinate complexes have been described by Sawyer (112).

ADP, histidine, bipyridyl, orthophenanthroline, and nitriloacetic acid are all chelators which have shown multiple, sometimes contradictory, effects on lipid oxidation in various systems. The first two are physiological chelators and the latter three are chelators known to be toxic to humans. Lipophilicity, singlet oxygen scavenging, hypervalent iron facilitation have been proposed as possible explanations for the actions of these chelators, but the mechanisms of their actions remain incompletely understood.

A characteristic of chelators which has received little general recognition is that HO$^{\bullet}$ and perhaps also lipid oxyl radicals, formed in the reaction cage of peroxide reduction, react with some chelators to form chelator free radicals which are themselves reactive. EDTA, for example, forms four different free radicals, most of which are strongly reducing and hence may be important determinants in the course of catalyzed oxidations (166). TRIS and phosphate (167) in buffers and desferrioxamine (168, 169; Schaich, K.M., manuscript in preparation) are known to form reactive radicals when attacked by HO$^{\bullet}$. These complexing agents are very likely to be both targets and initiators for lipid oxyl radicals.

Still another factor which may potentially have great importance for lipid oxidation in living systems is charge on chelators and on membrane surfaces. Surface charges

TABLE 3

Redox Potentials of Some Common Metal Complexes and Heme Compounds (refs. 162,163)

Complex	(V *vs* NHE)[a]	
	E^{o}	$E^{o'}$[b]
Bipyridyl	1.11	
1,10 (ortho)phenanthroline	1.06	
Fe^{3+}/Fe^{2+}	0.77	
Ferricyanide		0.36
Acetate, pH 5		0.34
Malonate, pH 4		0.26
Salicylate, pH 4		0.26
DTPA		0.126
EDTA		0.012
Oxalate		0.002
Pyrophosphate (PPi)		−0.14
8-Hydroxyquinoline		−0.15
Cyt c		0.254
Hemoglobin		0.144
Myoglobin		0.046
Cyt b_5 microsomes		0.02
Hematoporphyrin(pyridine)$_2$		0.004
Protoporphyrin IX(his)$_2$,		
pH 9.5		−0.138
pH 8.2 (borate)		−0.188
pH 7.0 (phosphate)		−0.226
Peroxidase (horseradish)		−0.271
Ferredoxin		−0.413

[a]Normal hydrogen electrode.

[b]Redox potential at pH 7 unless otherwise noted.

affect molecular access and binding as well as the dynamics of electron transfer reactions through lipid phases, and thus may be particularly important for porphyrin catalyses (170). Also, the electrostatic environment (the total effective charge) surrounding the binding site of a metal can markedly affect the redox potential and other thermodynamic properties of the metal (171). The net effect often results from a complex competition between several individual effects and thus may be difficult to predict. Nevertheless, these various effects of charge need to be understood and considered when interpreting mechanisms of metal action in complex systems.

Some very elegant studies have shown distinctly different behaviors in membranes or micelles incubated with Fenton reagents, depending on the surface charge of the vesicles (172–176). Negatively charged vesicles repelled metals, so direct initiation of lipids could not be effected. However, mutual repulsions of charged groups at the surface created more open molecular packing, so small molecules from the aqueous phase were able to penetrate the lipid layers and mediate reactions there. For example, $HO^{\cdot}$ generated near the surface was able to penetrate into the hydrophobic regions of the vesicles and initiate oxidation of lipids there, and this oxidation could be effectively inhibited by α-tocopherol and by water-soluble $HO^{\cdot}$ scavengers. In contrast, vesicles with negative surface charges bound metals, which reacted with traces of lipid hydroperoxides in the vesicles to initiate new oxidation chains. Water-soluble radical scavengers had little or no effect on this LOOH reaction, and tocopherol actually accelerated lipid oxidation in these vesicles because it recycled the Fe^{3+}, thus maintaining LOOH reductions. Effects of chelators in these systems could be explained by the changes in metal distribution and binding attributable to chelator charges (177).

SUMMARY

The five contemporary issues presented in this paper show clearly that metal catalyses of lipid oxidation in complex, often multiphasic or compartmentalized reaction systems are not straightforward. They often do not fit the classical electron transfer mechanisms for metal catalysis which have been accepted for decades. New understandings must be developed to more accurately explain the kinetics, mechanisms, and products of metal-catalyzed lipid oxidation in complex environments. Definitive evidence for intermediates in proposed mechanisms must be sought, and consideration must be given to all physical and chemical properties of reactants and reaction environments when interpreting mechanism from kinetic and product data. New integrative approaches and expertise from many different scientific disciplines will be necessary to elucidate the many factors affecting and controlling metal reactions in complex, multiphase systems.

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Redox Cycling of Iron and Lipid Peroxidation[1]

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Mechanisms of iron-catalyzed lipid peroxidation depend on the presence or absence of preformed lipid hydroperoxides (LOOH). Preformed LOOH are decomposed by Fe(II) to highly reactive lipid alkoxyl radicals, which in turn promote the formation of new LOOH. However, in the absence of LOOH, both Fe^{2+} and Fe^{3+} must be available to initiate lipid peroxidation, with optimum activity occurring as the Fe^{2+}/Fe^{3+} ratio approaches unity. The simultaneous availability of Fe^{2+} and Fe^{3+} can be achieved by oxidizing some Fe^{2+} with hydrogen peroxide or with chelators that favor autoxidation of Fe^{2+} by molecular oxygen. Alternatively, one can use Fe^{3+} and reductants like superoxide, ascorbate or thiols. In either case excess Fe^{2+} oxidation or Fe^{3+} reduction will inhibit lipid peroxidation by converting all the iron to the Fe^{3+} or Fe^{2+} form, respectively. Superoxide dismutase and catalase can affect lipid peroxidation by affecting iron reduction/oxidation and the formation of a (1:1) Fe^{2+}/Fe^{3+} ratio. Hydroxyl radical scavengers can also increase or decrease lipid peroxidation by affecting the redox cycling of iron.
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Lipid peroxidation is the process in which molecular oxygen is incorporated into unsaturated lipids (LH) to form lipid hydroperoxides (LOOH). However, the direct reaction of lipids with oxygen is spin-forbidden because the ground state of lipids is of singlet multiplicity whereas that of oxygen is of triplet multiplicity (1). Lipid peroxidation must therefore occur *via* reactions that by-pass the spin barrier between lipids and oxygen. These reactions are promoted by some type of "initiator" (I$^\bullet$) that overcomes the dissociation energy of an allylic bond and thus causes hydrogen abstraction and formation of a lipid alkyl radical (L$^\bullet$). Lipid alkyl radicals can rapidly add oxygen to form lipid peroxyl radicals (LOO$^\bullet$) which eventually liberate LOOH *via* hydrogen abstraction from a neighboring allylic bond (2).

$$I^\bullet + LH \rightarrow IH + L^\bullet \quad \text{initiation} \quad [1]$$

$$L^\bullet + O_2 \rightarrow LOO^\bullet \quad O_2 \text{ addition} \quad [2]$$

$$LOO^\bullet + LH \rightarrow LOOH + L^\bullet \quad \text{propagation} \quad [3]$$

Externally generated oxidants are required to initiate lipid peroxidation, but once started, the reaction will proceed *via* propagation to form new LOOH. Transition metals can substantially enhance the propagation of lipid peroxidation. For example, Fe^{2+} will reductively cleave LOOH to highly reactive alkoxyl (LO$^\bullet$) radicals, which in turn abstract hydrogen from lipids to form new lipid alkyl radicals (3). This reaction is referred to as LOOH-dependent lipid peroxidation (3).

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^\bullet + OH^- \quad [4]$$

$$LO^\bullet + LH \rightarrow LOH + L^\bullet \quad [5]$$

The realization that numerous diseases may involve lipid peroxidation as a common pathogenic mechanism (4) has prompted interest in identification of possible initiators (see Eq. 1). Both iron and partially reduced species of dioxygen have been implicated in the formation of initiators, although with very different interpretations concerning the mechanism of their involvement.

One very popular theory dictates that lipid peroxidation is initiated by $^\bullet$OH (5–8), the most potent oxidant that can be formed from oxygen ($E^\circ = 1.6$ V) (9). This is thought to occur *via* the Haber-Weiss reaction, that consists of $O_2^{-\bullet}$ dependent Fe^{3+} reduction and subsequent Fe^{2+}-catalyzed H_2O_2 cleavage to $^\bullet$OH (10).

$$O_2^{-\bullet} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad [6]$$

$$2O_2^{-\bullet} + 2H^+ \rightarrow 2H_2O_2 + O_2 \quad [7]$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^\bullet OH \quad [8]$$

Ferrous iron-promoted breakdown of H_2O_2 (Eq. 8) is best known as the Fenton reaction, and the Haber-Weiss reaction may alternatively be referred to as $O_2^{-\bullet}$ driven Fenton reaction, with this terminology illustrating that $O_2^{-\bullet}$ is required for the formation of both Fe^{2+} (*via* reduction of Fe^{3+}, Eq. 6) and H_2O_2 (*via* dismutation, Eq. 8) (10). According to this interpretation, the inhibition of lipid peroxidation by superoxide dismutase (SOD), catalase or hydroxyl radical ($^\bullet$OH) scavengers is viewed as supporting evidence of initiation of lipid peroxidation *via* Fenton chemistry and the $^\bullet$OH (6,7) As an alternate proposal for the initiation of lipid peroxidation however, some investigators have proposed that iron oxygen complexes can substitute for $^\bullet$OH in the initiation of lipid peroxidation, provided that both Fe^{2+} and Fe^{3+} are involved in the formation of this complex (11–20). This alternate interpretation implies that $O_2^{-\bullet}$ and H_2O_2 are required not to form $^\bullet$OH but rather to promote the Fe^{3+} reduction or Fe^{2+} oxidation from which the appropriate Fe^{2+}/Fe^{3+} initiating species originates. Consistent with this proposal, the effects of SOD, catalase and $^\bullet$OH scavengers have been found to vary depending on their effects on the iron valence state (15,20). Furthermore, $O_2^{-\bullet}$ and H_2O_2

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Abbreviations: ADP, adenosine diphosphate; EDTA, ethylenediaminetetraacetic acid; $^\bullet$OH, hydroxyl radical; L$^\bullet$, lipid alkyl radical; LH, unsaturated lipids; LOOH, lipid hydroperoxides; LOO$^\bullet$, lipid peroxyl radicals; MDA, malondialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $O_2^{-\bullet}$, superoxide; SOD, superoxide dismutase.

independent mechanisms for the redox cycling of iron and the initiation of lipid peroxidation have been described (18,20).

In this article we describe and discuss some mechanisms for the redox cycling of iron and their relevance to the initiation of lipid peroxidation by either $^{\bullet}OH$ or equally effective iron/oxygen complexes.

FERROUS IRON OXIDATION AND LIPID PEROXIDATION

Lipid peroxidation can be studied in incubations containing unchelated Fe^{2+} and either commercially available fatty acids or microsomal phospholipid liposomes. Ferrous iron *per se* may promote or not promote lipid peroxidation, depending on whether lipids contain preformed LOOH. In the presence of preformed LOOH, Fe^{2+} will catalyze what we have previously referred to as LOOH-dependent lipid peroxidation (3), a reaction resembling propagation rather than initiation mechanisms. In the absence of preformed LOOH, or when the concentration of LOOH is so low that they may not react with Fe^{2+} to an appreciable rate, H_2O_2 can be used to promote Fenton reaction and $^{\bullet}OH$ formation. We observed that in model systems including $FeCl_2$ and microsomal phospholipid liposomes with very little, if any, LOOH contamination, the inclusion of H_2O_2 promoted lipid peroxidation, as evidenced by formation of malondialdehyde (MDA) (Table 1). The reaction was inhibited by catalase, but the addition of $^{\bullet}OH$ scavengers either inhibited or stimulated MDA formation. These effects were not due to the ability of mannitol and benzoate to scavenge $^{\bullet}OH$, but could be explained by the ability of these compounds to interfere with Fe^{2+} oxidation. Thus, lipid peroxidation was inhibited by mannitol, which minimizes Fe^{3+} formation, whereas lipid peroxidation was stimulated by benzoate, which facilitates Fe^{3+} formation (see also Table 1). Collectively, these observations have led us to question the involvement of $^{\bullet}OH$ in lipid peroxidation and the effects of $^{\bullet}OH$ scavengers therein. We have proposed that: (i) Fe^{2+} dependent peroxidation of LOOH-free lipids is mediated by an oxidant that also requires Fe^{3+}, (ii) the role of H_2O_2 is that of oxidizing Fe^{2+} to Fe^{3+} rather than of serving as precursor of $^{\bullet}OH$, and (iii) chemicals referred to as $^{\bullet}OH$ scavengers must be used with great caution, since they can interfere with lipid peroxidation by modulating the rate of Fe^{2+} oxidation.

TABLE 1

Effects of Catalase, $^{\bullet}OH$ Scavengers and Detergents on Fe^{2+} and H_2O_2 Dependent Lipid Peroxidation[a,b]

System	Fe^{2+} oxidation (%)	nmol MDA/mL/min
Fe^{2+}	0	0.0
Fe^{2+}, H_2O_2	50	3.8
+ catalase	0	0.0
+ mannitol	30	0.7
+ benzoate	85	5.5
Fe^{2+}, H_2O_2, Lubrol-PX	53	6.1
+ catalase	0	0.0
+ mannitol	39	2.8
− benzoate	92	8.2

[a] Incubations contained microsomal phospholipid liposomes (1 μmol lipid phosphate/mL) ± 0.1% Lubrol-PX, in 50 mM NaCl, pH 7.0, 37°C. Where indicated, additions were made as follows: $FeCl_2$, 0.2 mM; H_2O_2, 0.1 mM; catalase, 400 U/mL; mannitol, 25 mM; benzoate, 25 mM. Ferrous iron oxidation was monitored as disappearance of 1,10-phenanthroline chelatable Fe^{2+} in lipid-free incubations and values are those determined at 30 sec. NaCl was chromatographed on Chelex-100 to remove contaminating metals, and catalase was chromatographed on Sephadex G-25 to remove the antioxidant thymol and other contaminants. MDA formation was determined by thiobarbituric acid test within the linear phase of the reactions (4–6 min.).

[b] Data taken in part from Minotti and Aust (15).

In principle, our skepticism concerning the participation of $^{\bullet}OH$ in lipid peroxidation agrees with the criticism that $^{\bullet}OH$ is a short-lived radical with diffusion-limited reactivity (21). As such, $^{\bullet}OH$ would not migrate from the aqueous phase of incubations to the hydrophobic membrane compartments where the allylic bonds of fatty acids are buried. For example, any $^{\bullet}OH$ generated in the aqueous bulk of incubations can react with residual Fe^{2+} and H_2O_2 (Eqs. 9–10) or can undergo bimolecular recombination to water (Eq. 11), thus self-precluding from reaction with lipids (8,9).

$$^{\bullet}OH + Fe^{2+} + H^+ \rightarrow Fe^{3+} + H_2O \quad [9]$$

$$^{\bullet}OH + H_2O_2{}^{\bullet} \rightarrow HO_2{}^{\bullet} + H_2O \quad [10]$$

$$^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2 \quad [11]$$

However, Schaich and Borg (8,22) have found that critical amounts of iron and H_2O_2 can partition from water into lipids. They have therefore proposed that Fenton reaction may take place in the lipids, so that "site specific" reaction of $^{\bullet}OH$ with juxtaposed allylic bonds would prevail over nonproductive side reactions in the aqueous phase. Viewed in this context, stimulation or inhibition of lipid peroxidation by benzoate or mannitol might simply reflect spurious and diverging effects of these compounds on $^{\bullet}OH$-dependent reactions that occur in the aqueous phase and either "consume" Fe^{2+} and H_2O_2 (*cf.* Eqs. 9–10) or evolve H_2O_2 (*cf.* Eq. 11). Mannitol and benzoate would affect lipid peroxidation by affecting the concentration of Fe^{2+} and H_2O_2 that migrate from water to lipids and promote the intramembranous formation of $^{\bullet}OH$.

In order to validate or disprove this hypothesis, we have studied Fe^{2+} and H_2O_2-dependent peroxidation of phospholipid liposomes dispersed in nonionic detergent Lubrol-PX. In this system, the detergent served to modify lipid configuration and facilitated the partitioning of both iron and $^{\bullet}OH$ scavengers from water to lipids (6,23). This would facilitate the formation of $^{\bullet}OH$ in closest proximity of the allylic bond, thus minimizing reactions of $^{\bullet}OH$ in the aqueous phase and nonspecific interferences of $^{\bullet}OH$ traps therein. As shown in Table 1, Lubrol-PX caused a substantial increase of lipid peroxidation, although it did not modify the extent of Fe^{2+} oxidation. This indicates that Lubrol-PX stimulates lipid peroxidation mainly by increasing the accessibility of iron to lipids. However, the effects of catalase, mannitol and benzoate on Fe^{2+} oxidation and formation of MDA were the same as observed in incubations lacking the detergent (see also Table 1). Therefore, we must reiterate the earlier contention that the initiation of lipid peroxidation by Fe^{2+} is mediated by

TABLE 2

Effect of Preincubating Fe^{2+} with H_2O_2 on Fe^{2+} Oxidation and Lipid Peroxidation[a,b]

Preincubation time (sec)	Fe^{2+} oxidation (%)	nmol MDA/mL/min
0	0	0.0
15	39	3.9
30	50	4.3
60	68	2.7
120	88	0.9
240	100	0.0

[a] Hydrogen peroxide (0.1 mM) and $FeCl_2$ (0.2 mM) were preincubated in 50 mM NaCl, pH 7.0, 37°C, for the time required to achieve the indicated extents of Fe^{2+} oxidation, measured as described in legend to Table 1. At the end of preincubation, catalase (400 U/mL) was included to scavenge any remaining H_2O_2, and microsomal phospholipid liposomes (1 μmol lipid phosphate/mL) were included to study lipid peroxidation.

[b] Data from Minotti and Aust (15).

oxidants that provide Fe^{3+}. Thus, in this system H_2O_2 must be available to ensure Fe^{2+} oxidation, whereas mannitol and benzoate will modulate lipid peroxidation by decreasing or increasing Fe^{3+} formation, irrespective of their ability to intercept $^{\bullet}OH$ in either aqueous or lipid environments.

To quantitate how much Fe^{3+} is required for the initiation of lipid peroxidation using Fe^{2+}, we studied liposome incubations containing various amounts of Fe^{2+} and Fe^{3+} generated by reacting Fe^{2+} with H_2O_2 for increasing periods of time. As shown in Table 2, the rate of lipid peroxidation was highest when approximately 50% of Fe^{2+} had been oxidized to Fe^{3+}. Lipid peroxidation did not occur when all of the iron remained in the reduced form nor when H_2O_2 had oxidized all the Fe^{2+} to Fe^{3+}. These results suggested to us that only a fraction of Fe^{2+} must be converted to Fe^{3+}, with optimum activity occurring as the Fe^{2+}/Fe^{3+} ratio approaches unity.

FERROUS IRON AUTOXIDATION AND LIPID PEROXIDATION

The autoxidation of Fe^{2+} may represent an alternate means to generate Fe^{3+} and thus initiate lipid peroxidation. Iron autoxidation consists of direct electron transfer from $Fe^{2+}+$ to molecular oxygen, leading to formation of $O_2^{-\bullet}$ and Fe^{3+} (Eq. 12). Alternatively, and perhaps more likely (1), two Fe^{2+} can autoxidize at the expense of one molecule of oxygen, thus causing the formation of H_2O_2 and two Fe^{3+} (Eq. 13).

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\bullet} \quad [12]$$

$$2Fe^{2+} + O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O_2 \quad [13]$$

In order for iron to autoxidize, the reduction potential of the Fe^{2+}/Fe^{3+} couple must be lower than that of the $O_2^{-\bullet}/O_2$ couple (−0.33 V) (24). It is generally assumed that the redox potential of Fe^{2+}/Fe^{3+} couple is −0.77 V (25) and that chelators with oxygen donor atoms and greater affinity for Fe^{3+} lower this value (26). However, ^{-}OH ligation of the iron may *per se* lower the redox potential of Fe^{2+} by about 0.66 V, thus making the autoxidation of "free" Fe^{2+} theoretically feasible (1). However, even in such a case, chelators will dictate the rate of iron autoxidation. For example, unchelated Fe^{2+} and ethylenediaminetetraacetic acid (EDTA) chelated Fe^{2+} have a similar redox potential (−0.11 V) (1), yet $FeSO_4$ or $FeCl_2$ will autoxidize very slowly whereas EDTA/Fe^{2+} will autoxidize very rapidly, provided that these reactions are studied at neutral pH and in an inert solution like NaCl (27).

It is a frequent misconception that unchelated Fe^{2+} autoxidizes rapidly, but this contention does not take into account that several laboratory buffers chelate iron and modify its redox chemistry, ultimately favoring Fe^{2+} autoxidation. Phosphate buffers are perhaps the most effective in causing Fe^{2+} autoxidation (1), and this may explain some conflicting reports in the literature. For example, we have already mentioned that unchelated Fe^{2+} cannot peroxidize LOOH-free liposomes incubated in NaCl, but others have shown that Fe^{2+} may quite effectively promote the peroxidation of liposomes incubated in phosphate buffers (28). Irrespective of different LOOH contaminations of liposomes prepared in different laboratories, it is quite possible that lipid peroxidation in phosphate-buffered incubations is mediated by Fe^{2+} autoxidation and formation of the Fe^{3+} also required for initiation rather than by decomposition of LOOH by Fe^{2+} alone.

There are some basic aspects of ligand-affected iron autoxidation that must be kept in mind for rigorous interpretation of lipid peroxidation experiments. First of all, iron autoxidation increases with the chelator/Fe^{2+} ratio, with the extent of autoxidation strictly depending on the chelator being used. For example, EDTA/Fe^{2+} autoxidizes much more rapidly than citrate/Fe^{2+} and the autoxidation of approximately 50–70% of the iron will therefore occur at very different chelator/Fe^{2+} ratios (1:1 for EDTA/Fe^{2+} *vs* 20:1 for citrate/Fe^{2+}) (16,27). Second, in the presence of ligands Fe^{2+} autoxidation may not be significantly inhibited by catalase (16,20,27). Direct consequences of these mechanisms of iron autoxidation on the initiation of lipid peroxidation are exemplified in Table 3. The data indicate that both citrate/Fe^{2+} and

TABLE 3

The Effects of Chelators on Iron oxidation and Lipid Peroxidation[a,b]

Chelate	Fe^{2+} oxidation (%)		nmol MDA/mL/min	
	− catalase	+ catalase	− catalase	+ catalase
EDTA/Fe^{2+}				
(1:1)	75	63	1.4	1.3
(20:1)	100	100	0.0	0.0
Citrate/Fe^{2+}				
(1:1)	19	16	0.3	0.4
(20:1)	63	48	1.0	1.2

[a] Incubations contained microsomal phospholipid liposomes (1 μmol lipid phosphate/mL) in 50 mM NaCl, pH 7.0, 37°C. Reactions were started by addition of chelates at different chelator/$FeCl_2$ ratios. The concentration of Fe^{2+} was 200 μM. Where indicated, catalase (400 U/mL) was included. Iron oxidation was measured as described in the legend to Table 1 and values are those determined at 30 sec. MDA formation is expressed as initial rates.

[b] Data from Minotti and Aust (16,27).

EDTA/Fe^{2+} catalyzed lipid peroxidation, yet the EDTA/Fe^{2+} ratio had to be 1:1 and the citrate/Fe^{2+} ratio had to be 20:1. This is because lipid peroxidation occurred when only a critical fraction of Fe^{2+} was converted to Fe^{3+}. Very little, if any, lipid peroxidation occurred with either 20:1 EDTA/Fe^{2+} or 1:1 citrate/Fe^{2+} ratios, which caused too extensive or too moderate autoxidation, respectively, and thus shifted the Fe^{2+}/Fe^{3+} ratios toward values that were not permissive to lipid peroxidation (see also Table 3). By contrast, EDTA and citrate/Fe^{2+} dependent lipid peroxidation were largely insensitive to the addition of catalase, as were the autoxidation rates of the two chelates (Table 3). This underscored a substantial difference from systems in which H_2O_2 dependent and hence catalase-inhibitable oxidation of unchelated Fe^{2+} was mandatory to form the necessary Fe^{3+} (*cf.* Table 1).

The observation that catalase cannot affect iron autoxidation must be examined from different viewpoints. One possibility might be that the vast majority of Fe^{3+} forms *via* Eqs. 12–13, with very minor contribution by subsequent reactions of residual Fe^{2+} and the H_2O_2 formed by oxygen reduction. Another possibility might be that H_2O_2 does participate in Fe^{3+} formation yet the access of catalase to the site of reaction between Fe^{2+} and H_2O_2 is made sterically unfavorable by some type of "cage effect" (29) of the chelator being used.

Although speculative in nature, this latter possibility should not be neglected, because we have found that exogenously added H_2O_2 can react with citrate/Fe^{2+} and that simultaneous addition of catalase prevents this reaction (16). Therefore, one may not rule out that ligand-affected Fe^{2+} autoxidation would be paralleled by H_2O_2 dependent Fe^{2+} oxidation (see Table 3). Irrespective of any further speculation on the difference between exogenously added or endogenously formed H_2O_2 for reaction with chelated iron, it is noteworthy that the addition of H_2O_2 can either stimulate or inhibit citrate/Fe^{2+} dependent lipid peroxidation, depending on the citrate/Fe^{2+} ratio. With a low citrate/Fe^{2+} ratio and very moderate Fe^{2+} autoxidation, the addition of H_2O_2 was found to expedite Fe^{3+} formation and approach Fe^{2+}/Fe^{3+} ratios that favored lipid peroxidation (see Table 4). With a high citrate/Fe^{2+} ratio and more extensive Fe^{2+} autoxidation, the addition of H_2O_2 caused the formation of too much Fe^{3+}, thereby shifting the Fe^{2+}/Fe^{3+} ratio toward values that were not permissive to lipid peroxidation (Table 4). In either case, the simultaneous addition of catalase prevented the reaction of H_2O_2 with citrate/Fe^{2+}, changed the Fe^{2+}/Fe^{3+} ratio, and either stimulated or inhibited lipid peroxidation. These results confirmed the role of H_2O_2 in forming Fe^{3+} from Fe^{2+}, with ultimate effects on lipid peroxidation depending on the ratio of Fe^{2+}/Fe^{3+}.

Some investigators have noted that unchelated Fe^{2+} can very rapidly promote lipid peroxidation of "intact" microsomes, irrespective of the presence or absence of an oxidant like H_2O_2 or chelators favoring the autoxidation of Fe^{2+} (28). The occurrence of lipid peroxidation in incubations containing microsomes and Fe^{2+} but neither H_2O_2 or chelators has been taken as evidence against the requirement for both Fe^{2+} and Fe^{3+}, which was first observed in liposomal systems. In this respect, Table 5 shows that all of the exogenously added $FeSO_4$ was recovered from incubations containing liposomes but not from incubations containing microsomes from which these same liposomes have been prepared. Microsome-induced ferrous iron disappearance was inhibited by anaerobiosis but not by catalase, as was the autoxidation of EDTA/Fe^{2+} or citrate/Fe^{2+} (*cf.* Table 3 and Table 5). It is difficult to propose that Fe^{2+} would react with preformed LOOH of "intact" microsomes but not of their extracted lipids. We would rather propose that microsomes, but not liposomes, favor reaction of Fe^{2+} with oxygen and formation of the Fe^{3+} required for initiation of lipid peroxidation (see Table 5). Studies are in progress to identify the nonlipid microsome components that cause iron oxidation. Irrespective of the precise nature of these components, however, it is imperative to recognize microsome-induced Fe^{2+} oxidation as an additional mechanism for Fe^{3+}

TABLE 4

Effects of H_2O_2 on Citrate/Fe^{2+} Dependent Lipid Peroxidation[a,b]

Citrate/Fe^{2+} ratio	System	nmol MDA/mL/min	% Change
1:1	Complete	0.3	
	+ H_2O_2	1.3	+ 333
	+H_2O_2 + catalase	0.4	+33
20:1	Complete	1.4	
	+ H_2O_2	0.3	−79
	+H_2O + catalase	1.3	−7

[a] Complete systems contained citrate (at the indicated citrate/Fe^{2+} ratios) and microsomal phospholipid liposomes (1 μmol lipid phosphate/mL) in 50 mL NaCl, pH 7.0, 37°C. Where indicated, 10 μM H_2O_2 and catalase (400 U/mL) were included in the reaction mixtures. Fe^{2+} concentration was 200 μM. Values of MDA formation are given as initial rates.

[b] Data from Minotti and Aust (16).

TABLE 5

Iron Oxidation and Lipid Peroxidation: Comparison of Microsomal *vs* Liposomal Systems[a]

System	Fe^{2+} oxidation (%)	nmoles MDA/mL/min
Fe^{2+} + liposomes	4	0.0
Fe^{2+} + microsomes	48	1.2
Fe^{2+}, microsomes, catalase	46	1.2
Fe^{2+} + microsomes (anaerobic)	3	0.1

[a] Incubations contained $FeSO_4$ (60 μM) and either microsomes (0.6 mg protein/mL) or microsomal phospholipid liposomes (0.6 μmol lipid phosphate/mL) in 50 mM NaCl, pH 7.0, 37°C. In experiments with microsomes, iron oxidation was measured at 5 min as disappearance of bathophenanthroline chelatable Fe^{2+} in 105,000 × *g* supernatants of incubations, as described (20). In experiments with liposomes, aliquots of incubations were mixed with bathophenanthroline and formation of bathophenanthroline/Fe^{2+} complex was determined spectrophotometrically upon addition of Tween 80 to eliminate turbidity, as described in (17). Where indicated, incubations were made anaerobic by means of argon plus glucose (5 mM) and glucose oxidase (10 U/mL); catalase was also included to scavenge H_2O_2 produced by glucose oxidase reaction. Microsomes were calcium-aggregated and chromatographed on Sepharose CL-2B to remove contaminants like SOD, catalase and ferritin (see also ref. 20). Values of MDA formation are given as initial rates.

formation and lipid peroxidation. This will aid the understanding and interpretation of results obtained by different investigators under various experimental conditions.

FERRIC IRON REDUCTION AND LIPID PEROXIDATION

Lipid peroxidation can also be studied in incubations containing ferric iron and reductants. One very popular system of lipid peroxidation relies on xanthine oxidase and adenosine diphosphate (ADP)/Fe^{3+} (30). The xanthine oxidase reaction generates $O_2^{-\bullet}$ as a reductant for Fe^{3+}. However, the xanthine oxidase reaction also generates H_2O_2, either *via* $O_2^{-\bullet}$ dismutation or *via* the direct two-electron reduction of molecular oxygen (31). Therefore, xanthine oxidase dependent generation of an iron reductant is accompanied by the generation of an iron oxidant. Keeping in mind that lipid peroxidation requires both Fe^{2+} and Fe^{3+}, one can easily anticipate that xanthine oxidase and ADP/Fe^{3+} dependent lipid peroxidation will be inhibited by SOD, which prevents Fe^{3+} reduction, and will be enhanced by catalase, which prevents Fe^{2+} oxidation back to Fe^{3+} (Table 6). We have determined that xanthine oxidase dependent generation of $O_2^{-\bullet}$ at a rate of approximately 50 μM/min will reduce 100 μM ADP/Fe^{3+} at a rate of 6 or 22 μM/min, depending on whether catalase is omitted or included to scavenge H_2O_2, respectively (32). This quantitation confirms that the inclusion of xanthine oxidase with Fe^{3+} rapidly makes both Fe^{3+} and Fe^{2+} available to lipids, especially when catalase is included to prevent Fe^{2+} reoxidation. The relatively high stoichiometry of $O_2^{-\bullet}$ generation *vs* Fe^{2+} formation (~5:1) that persists even in the presence of catalase indicates that some Fe^{2+} invariably oxidizes to Fe^{3+}. It is possible that ADP/Fe^{3+} reduction results in the formation of an ADP/Fe^{2+} complex, which in turn autoxidizes in a catalase-insensitive manner. Nonetheless, it should also be noted that ADP/Fe^{2+} autoxidizes quite moderately as compared to other chelates, *e.g.*, citrate/Fe^{2+} or EDTA/Fe^{2+} (*cf.* refs. 16,18,27). Therefore ADP/Fe^{2+} autoxidation may not preclude formation of an appropriate Fe^{2+}/Fe^{3+} ratio. Another possibility is that $O_2^{-\bullet}$ may also serve as an oxidant for Fe^{2+}, thereby maintaining iron within a continuous $Fe^{3+} \rightarrow Fe^{2+} \rightarrow Fe^{3+}$ cycle (1,32).

Thomas and Aust (33) and Saito *et al.* (34) have described ferritin- and transferrin-dependent lipid peroxidation systems in which xanthine oxidase is required to form $O_2^{-\bullet}$ and promote the reductive release of iron from these proteins. As a general feature, rates of iron release are less than one-tenth or one-fifth of rates of ADP/Fe^{3+} reduction (*cf.* refs. 32–34). Therefore, catalase must be included to prevent H_2O_2 from causing the complete oxidation of the released Fe^{2+} and inhibiting the formation of a suitable Fe^{2+}/Fe^{3+} ratio. The Fe^{3+} required to combine with Fe^{2+} and initiate lipid peroxidation will form *via* catalase-insensitive autoxidation of the released Fe^{2+}, which is favored by ADP included to facilitate iron release, perhaps *via* disruption of the ternary complex of iron-binding proteins (34), and also to chelate the released iron (33). However, excess ADP will cause the autoxidation of too much Fe^{2+}, thereby shifting the Fe^{2+}/Fe^{3+} ratio toward values that are not permissive to lipid peroxidation. As a result of the equilibrium between Fe^{2+} release and Fe^{2+} autoxidation, Saito *et al.* (34) have shown that xanthine oxidase and transferrin dependent lipid peroxidation is enhanced by low concentrations of ADP but inhibited by high concentrations of this same chelator, irrespective of the presence or absence of catalase to remove H_2O_2 (34).

It has been suggested that $O_2^{-\bullet}$ dependent Fe^{3+} reduction and lipid peroxidation can also be achieved by replacing xanthine oxidase with "autoxidizable" compounds like thiols and ascorbate (35,36). However, others have shown that "autoxidation" of thiols and ascorbate at the expense of molecular oxygen is neither thermodynamically or kinetically feasible unless iron is present to serve as direct one electron acceptor (1,18,27,37). Thus, ascorbate or thiol oxidation is mediated by direct electron addition to Fe^{3+}. Subsequent reactions of Fe^{2+} with oxygen can eventually form $O_2^{-\bullet}$. In agreement with this interpretation, Table 6 indicates that replacing xanthine oxidase with glutathione or ascorbate resulted in SOD-insensitive lipid peroxidation, indicative of $O_2^{-\bullet}$ independent ADP/Fe^{3+} reduction. Moreover, results presented in Table 6 indicate that catalase did not inhibit ascorbate or glutathione dependent lipid peroxidation, perhaps indicating either that ADP/Fe^{2+} autoxidized so slowly that H_2O_2 could not accumulate to oxidize more Fe^{2+} and prevent initiation; or that H_2O_2 was formed in such a way that it could not be intercepted by catalase. Overall, it seems that lipid peroxidation is mediated by ADP/Fe^{3+} reduction and that ADP/Fe^{2+} would in turn combine with residual ADP/Fe^{3+} to form some type of Fe^{2+}/Fe^{3+} complex.

Ascorbate is an effective iron reductant, therefore relatively minor changes in its concentration will result in substantial changes in the Fe^{2+}/Fe^{3+} ratio and lipid peroxidation. For example, 25 μM ("low") ascorbate reduces some Fe^{3+} to Fe^{2+} and promotes lipid peroxidation, whereas 50 μM ("high") ascorbate reduces too much

TABLE 6

Ferric Iron-Dependent Lipid Peroxidation: Comparison of Xanthine Oxidase *vs* Glutathione and Ascorbate Systems[a]

System	nmol MDA/mL/min	% Change
Xanthine oxidase[b]	1.2	
+ SOD	0.3	−75
+ catalase	1.6	+33
Glutathione[c]	1.3	
+ SOD	1.4	+8
+ catalase	1.3	—
Ascorbate[d]	0.7	
+ SOD	0.7	—
+ catalase	0.7	—

[a] Incubations contained microsomal phospholipid liposomes (1 μmol lipid phosphate/mL) in 50 mM NaCl, pH 7.0, 37°C. Lipid peroxidation was assayed as malondialdehyde (MDA) formation (3).

[b] System contained 0.11 mM ADP, 0.1 mM $FeCl_2$, 0.33 mM xanthine and 0.05 U/mL xanthine oxidase. SOD and catalase were added at 100 and 400 U/mL, respectively (27). Xanthine oxidase was chromatographed on Sephadex G-25 to remove ammonium sulfate and other contaminants.

[c] System contained 0.11 mM ADP, and 0.1 mM $FeCl_3$ and 1 mM glutathione. SOD and catalase were added at 100 and 400 U/mL, respectively (27).

[d] System contained 0.25 mM ADP, 0.05 mM $FeCl_3$ and 12.5 μM ascorbate. SOD and catalase were added at 15 U/mL (17).

TABLE 7

Ascorbate-Dependent Lipid Peroxidation[a,b]

System	nmol MDA/mL/min	% Change
"Low" ascorbate	0.77	
+ H_2O_2	0.60	−22
"High" ascorbate	0.16	
+ H_2O_2	0.92	+475

[a] Incubations contained microsomal phospholipid liposomes (1 μmol lipid phosphate/mL) and ADP/Fe^{3+} (250 μM-50 μM) in 50 mM NaCl, pH 7.0, 37°C. Reactions were started by addition of either "low" (25 μM) or "high" (50 μM) ascorbate and lipid peroxidation was assayed as malondialdehyde (MDA) formation (3). Where indicated, 50 μM H_2O_2 was also included in the reaction mixtures.
[b] Data from Miller and Aust (18).

Fe^{3+} to Fe^{2+} and inhibits lipid peroxidation (Table 7). Very similar observations have been made with thiols like cysteine or dithiothreitol, which also promote moderate to excessive iron reduction upon minor increase in their concentration (38). Glutathione is a much weaker reductant than is ascorbate, cysteine or dithiothreitol (*cf.* refs. 18,38). Therefore, glutathione may not achieve the complete reduction of iron and inhibition of lipid peroxidation, even when it is used at rather high concentrations. Not surprisingly, both glutathione and ascorbate were capable of promoting lipid peroxidation, yet the concentration of glutathione was approximately 80-fold that of ascorbate (*cf.* legend to Table 6). From another viewpoint, concentrations of glutathione favoring lipid peroxidation were 20-fold those of ascorbate that inhibited lipid peroxidation (*cf.* Tables 6 and 7). One can use H_2O_2 to modulate the balance of reduced *vs* oxidized iron and either inhibit or stimulate lipid peroxidation. For example, with "low" concentration of ascorbate and partial Fe^{3+} reduction, the addition of H_2O_2 will reconvert more Fe^{2+} to Fe^{3+}, thereby inhibiting lipid peroxidation (Table 7). With "high" concentration of ascorbate and extensive or eventually complete Fe^{3+} reduction, the addition of H_2O_2 will oxidize some Fe^{2+} to Fe^{3+}, thereby stimulating lipid peroxidation (Table 7). These experiments indicate that reductants/oxidants can facilitate or prevent lipid peroxidation by virtue of their ability to modulate the Fe^{2+}/Fe^{3+} ratio. In particular, the effects of H_2O_2 on ascorbate dependent lipid peroxidation indicate that its ability to stimulate or inhibit will solely depend on how much iron is maintained in the Fe^{3+} form to achieve the formation of appropriate Fe^{2+}/Fe^{3+} ratios.

POSSIBLE EXPLANATIONS FOR THE REQUIREMENT FOR BOTH FE^{2+} AND FE^{3+}

The results described thus far indicate that the simultaneous availability of Fe^{2+} and Fe^{3+} determines whether lipid peroxidation will occur. The diverging effects of $^{\bullet}OH$ scavengers and the observations that SOD and catalase can either stimulate, inhibit or not affect lipid peroxidation apparently rule out any major role for $^{\bullet}OH$ in initiation of lipid peroxidation. These and other considerations, such as the previously mentioned diffusion-limited reactivity of $^{\bullet}OH$, have led Bucher *et al.* (11), Minotti and Aust (15,16,27) and Braughler *et al.* (12–14) to propose that lipid peroxidation is mediated by some type of iron/oxygen complex comprised of both Fe^{2+} and Fe^{3+}, and perhaps oxygen. All attempts by our laboratories to characterize this elusive complex have been unsuccessful so far. But studies of Co^{2+} or Co^{3+} dependent reactions have shown that the formation of oxygen-bridged complexes of reduced and oxidized metals is feasible (39).

Other investigators (40,41) have proposed that the iron/oxygen complex involved in lipid peroxidation can be best described as the perferryl ion ($Fe^{3+}O_2^{-\bullet}$), which forms *via* the following reactions of Fe^{2+} with oxygen, or the reduction of Fe^{3+} with $O_2^{-\bullet}$.

$$Fe^{2+} + O_2 \rightleftharpoons Fe^{2+}O_2 \rightleftharpoons Fe^{3+} + O_2^{-\bullet} \qquad [14]$$

Interestingly, Goddard and Sweeney (42) and Ursini *et al.* (43) have noted that the requirement for both Fe^{2+} and Fe^{3+} may underlie the initiation by perferryl iron rather than by ferrous/dioxygen/ferric complex(es) for at least two reasons. First, perferryl iron formation and reaction with lipids best occur with chelators and chelator/Fe^{2+} ratios that favor only moderate Fe^{2+} autoxidation and generate an equilibrium between $Fe^{2+}O_2$ and $Fe^{3+}O_2^{-\bullet}$ (43). Second, excess Fe^{2+} would compete with lipids as electron donors for $Fe^{3+}O_2^{-\bullet}$, thereby inhibiting hydrogen abstraction and lipid peroxidation (42,43). This proposed competition between lipids and excess Fe^{2+} for perferryl ion seems consistent with our findings that lipid peroxidation is maximal when critical amounts of Fe^{2+} oxidize to Fe^{3+} (Tables 3–5). Therefore, the perferryl ion model of lipid peroxidation is intriguing and deserves experimental attention. However, excellent rates of lipid peroxidation have been observed in incubations containing H_2O_2 and unchelated Fe^{2+}, which may not autoxidize to generate the equilibrium of $Fe^{2+}O_2$ with $Fe^{3+}O_2^{-\bullet}$ (Tables 1 and 2). Furthermore, SOD did not inhibit lipid peroxidation induced by Fe^{3+} plus ascorbate or glutathione (Table 6) nor that induced by Fe^{2+} chelates (11,16) perhaps indicating that an $O_2^{-\bullet}$-centered radical like perferryl ion may not be involved in either of these systems. Xanthine oxidase dependent lipid peroxidation is inhibited by SOD (Table 5) because Fe^{3+} reduction is mediated by $O_2^{-\bullet}$ in this system, regardless of whether Fe^{2+} is subsequently utilized to form the perferryl ion or a ferrous/dioxygen/ferric complex. Overall, there seem to be some positive but also some negative considerations to support the initiation by perferryl ion rather than by a ferrous/dioxygen/ferric complex.

Schaich and Borg (8) have proposed that irrespective of the nature of the reactive species which abstracts hydrogen from lipids, excess Fe^{2+} would compete as electron donors for $LOO^{\bullet}$ and $LO^{\bullet}$, thereby inhibiting both the formation of first LOOH and subsequent LOOH dependent propagation. As an additional criticism, it has been emphasized that most lipid peroxidation systems rely on high concentrations of iron, which not only fail to reproduce *in vivo* situations but also exaggerate the "antioxidant" effects by Fe^{2+} (8). The validity of this criticism is somehow weakened by previous observations that the requirement for both Fe^{2+} and Fe^{3+} is evident in ferritin and transferrin dependent systems, *i.e.*, systems mediated by physiological iron storage proteins *via* the reductive mobilization and subsequent autoxidation of

very low amounts of Fe^{2+} (33,34). We therefore conclude that Fe^{2+} oxidation is required not to decrease the concentration of Fe^{2+} as an antioxidant, but rather to increase the concentration of some type of oxidant that requires Fe^{3+}. Similar observations have recently been made for the initiation of lipid peroxidation by nonheme iron embedded in the microsomal milieu (20). However, lack of information on the precise structure of microsome-bound iron does not justify extensive discussion of these latter observations in the present article.

The importance of attaining an optimal Fe^{2+}/Fe^{3+} ratio in the initiation of lipid peroxidation was originally proposed and reiterated by relatively few investigators (11-16,27). An overview of the recent literature, however, reveals that similar results have been obtained by other research groups (44-47). Nevertheless, the mechanism of lipid peroxidation remains a matter of controversy. For example, it is certainly true that lipid peroxidation can be promoted by the direct addition of appropriate amounts of Fe^{2+} and Fe^{3+} yet Aruoma *et al.* (28) have found that Pb^{2+} and Al^{3+} can replace Fe^{3+} in the initiation of lipid peroxidation. This latter finding has been taken as evidence against the initiation of lipid peroxidation by a specific Fe^{2+}/Fe^{3+} complex. However, these investigators did not investigate the effects of Pb^{2+} or Al^{3+} on Fe^{2+} oxidation or the subsequent Fe^{2+}/Fe^{3+} ratio. This report also did not address the usual hypothesis that the $^{\bullet}OH$ was responsible for lipid peroxidation. It should be noted, however, that Pb^{2+} and Al^{3+} have also been found to inhibit the lipid peroxidation promoted by ADP/Fe^{3+} and either ascorbate or the reduced nicotinamide adenine dinucleotide phosphate (NADPH) dependent microsomal electron transport system, and that the reasons for such discrepancies have remained unexplained (28). Thus, the participation of Al^{3+} and Pb^{2+} in iron-dependent lipid peroxidation remains confined in very narrowed experimental conditions, whereas the formation of some type of Fe^{2+}/Fe^{3+} complex appears to mediate the initiation of lipid peroxidation in several model systems. We believe that the identification of intracellular low molecular weight iron complexes and the characterization of the mechanisms by which the cell regulates the redox state of these complexes and perhaps their interaction with other metals remain absolute prerequisites to further investigations in this field.

In conclusion, we have presented evidence that lipid peroxidation is mediated by some type of Fe^{2+}/Fe^{3+} complex which can be formed by Fe^{3+} reduction or by Fe^{2+} oxidation, and that these latter reactions can occur and be affected by various mechanisms.

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ARTICLE

Peroxide Dependent and Independent Lipid Peroxidation: Site-Specific Mechanisms of Initiation by Chelated Iron and Inhibition by α-Tocopherol[1]

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Peroxidation of linoleic acid (LA) was catalyzed by Fenton reagent (H_2O_2 and Fe^{2+}) in positively charged tetradecyltrimethylammonium bromide (TTAB) micelles, but not in negatively charged sodium dodecylsulfate (SDS) micelles. However, more hydroxyl radicals formed *via* the Fenton reaction were trapped by *N-t*-butyl-α-phenylnitrone (PBN) in SDS micelles than in TTAB micelles. Generation of linoleic acid alkoxy (LO) radicals by Fe^{2+} *via* reductive cleavage of linoleic acid hydroperoxide (LOOH) resulted in peroxidation of LA and formation of PBN-LO· adducts in SDS micelles, but not in TTAB micelles. This LOOH dependent lipid peroxidation could be catalyzed in TTAB micelles in the presence of a negatively charged iron chelator, nitrilotriacetic acid (NTA). LO radicals formed by the LOOH dependent Fenton reaction were also trapped by PBN at the surface of TTAB micelles in the presence of NTA, but not in its absence. The consumption of a spin probe, 16-(*N*-oxyl-4,4′-dimethyloxazolidin-2-yl)stearic acid (16-NS) during the LOOH dependent Fenton reaction in the presence of NTA was higher in TTAB micelles of LA than in those of lauric acid (LauA), although the rates and amounts of LO radicals formed in the two types of fatty acid micelles were similar. The rates of 5-NS consumption in LA and LauA micelles were almost the same, and were lower than the rate of 16-NS in LA micelles. NTA-Fe^{2+} initiated peroxidation of LA in TTAB micelles without a lag time in the presence of LOOH, but after a lag period, peroxidation occurred without LOOH. α-Tocopherol inhibited peroxidation of LA catalyzed by Fenton reagent by scavenging OH radicals in TTAB micelles. In contrast, α-tocopherol enhanced free Fe^{2+} induced LOOH dependent lipid peroxidation through the regeneration of Fe^{2+} in SDS micelles. However, it inhibited NTA-Fe^{2+} induced LOOH dependent lipid peroxidation in TTAB micelles. The rate and amount of α-tocopherol oxidized by the Fe^{2+} induced, H_2O_2 dependent Fenton reaction were almost the same in TTAB micelles of LA and LauA. The oxidation of α-tocopherol by the NTA-Fe^{2+} induced, LOOH dependent Fenton reaction was greater and faster in LA micelles than in LauA micelles, although the rates of LO radical production in the two types of micelles were the same. During NTA-Fe^{2+} induced, LOOH dependent lipid peroxidation, α-tocopherol inhibited more effectively the consumption of 16-NS than 5-NS. The results are discussed in relation to the location of iron, the unsaturated bonding region of LA, the OOH group of LOOH, the radical trapping site of PBN, the spin sites of 5-NS and 16-NS, and the phenolic hydroxyl group of α-tocopherol in micelles with different charges.
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Lipid peroxidation is generally thought to cause functional abnormalities and pathological changes in biological systems. Transition metals, such as iron, are involved in the production of reactive oxygen species, such as the hydroxyl radical (·OH), lipid alkoxy radical (RO·), and the iron-oxygen species responsible for initiation of lipid peroxidation. The ·OH and RO· are derived from hydrogen peroxide (H_2O_2) and lipid hydroperoxide (ROOH), respectively, by Fenton and Fenton-like reactions:

$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + Fe^{3+} + OH^- \quad [1]$$

$$ROOH + Fe^{2+} \rightarrow RO\cdot + Fe^{3+} + OH^- \quad [2]$$

Ferrous chelates alone also initiate lipid peroxidation without requiring hydroperoxides such as H_2O_2 or ROOH, and the initiating species may be a perferryl (1) or ferryl (2) ion or a ferrous-ferric-oxygen (3,4).

Vitamin E (tocopherol), well known as one of the most effective natural lipophilic antioxidants, scavenges ·OH and free lipid radicals such as RO· and hydroperoxy radicals (ROO·).

Samuni *et al.* (5) proposed a "site-specific" Fenton mechanism in which the binding of a transition metal to the biological target is a prerequisite for OH radical mediated cell damage. Schaich and Borg (6) also suggested that the solubility of iron complexes in the lipid phase of membranes is a critical determinant of the catalytic effectiveness to initiate lipid peroxidation. Recently, we confirmed the "site-specific" mechanism of initiation by free and chelated iron, and the inhibition by α-tocopherol of peroxide dependent and independent peroxidation of LA in positively and negatively charged micelles (7–10). In this paper, we describe the dependence of the mechanism on the location of the iron, the unsaturated bonding region of LA, the OOH group of LOOH, and the OH group of α-tocopherol in micellar membranes. The results are discussed with reference to the site of the ·OH, LO· and LOO· and to their scavenging by α-tocopherol.

MATERIALS AND METHODS

Materials. 5- and 16-(*N*-oxy-4,4′-dimethyloxazolidin-2-yl)-stearic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). *N-t*-Butyl-α-phenylnitrone was from

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Abbreviations: ESR, electron spin resonance; LA, linoleic acid; LauA, lauric acid; LOOH, linoleic acid hydroperoxide; 5-NS, 5-(*N*-oxyl-4,4′-dimethyloxazolidin-2-yl)stearic acid; 16-NS, 16-(*N*-oxyl-4,4′-dimethyloxazolidin-2-yl)stearic acid; NTA, nitrilotriacetic acid; PBN, *N-t*-butyl-α-phenylnitrone; SDS, sodium dodecylsulfate; α-Toc, α-tocopherol; TPP, triphenylphosphine; TTAB, tetradecyltrimethylammonium bromide; UV, ultraviolet.

Sigma Chemical Co. (St. Louis, MO). Nitrilotriacetic acid and lauric acid were obtained from Nakarai Chemicals (Tokyo, Japan). Linoleic acid, triphenylphosphine and α,α′-dipyridyl were purchased from Wako Pure Chemical Industries (Tokyo, Japan). α-Tocopherol (>99% pure) was supplied by Eisai Pharmaceutical Co. (Tokyo, Japan). All other reagents were of analytical grade.

Preparation of LOOH. LOOH was prepared by the method of O'Brien (11) and purified by column chromatography as described by Gamage *et al.* (12).

Preparation of fatty acid micelles. Linoleic acid or lauric acid was dispersed in the form of mixed micelles with TTAB or SDS as described previously (9). Briefly, a solution of 25 μmol LA or LauA in chloroform was placed in a test tube, and the solvent was evaporated under nitrogen. The lipid film was dissolved in 5 mL of 50 mM TTAB or SDS solution, vortexed and then sonicated in a Branson sonic bath. When necessary, contaminating LOOH in LA was reduced to the corresponding LOH by treatment of LA with TPP in chloroform solution (13) just before the preparation of micelles.

Lipid peroxidation assay. Lipid peroxidation was measured by monitoring oxygen consumption with a Clark type electrode (8). Consumption was calculated assuming an oxygen concentration of 253 nmol/mL in the initial incubation mixture at 25°C. The pH of the mixture was adjusted by HCl or NaOH. The concentrations of the components of the reaction mixtures and the incubation conditions are given in the figure legends. LOOH formed was also determined iodometrically by the method of Buege and Aust (14) with slight modification.

Measurement of ferrous iron concentration. Ferrous iron was determined colorimetrically with α,α′-dipyridyl (15). Forty μL of 375 mM α,α′-dipyridyl solution in ethanol was added to 3 mL of incubation mixture, and absorbance at 520 nm was measured.

Measurement of α-tocopherol. Oxidation of α-tocopherol was assayed by measuring the change in ultraviolet (UV) absorbance as reported previously (16).

Measurement of electron spin resonance (ESR) spectra. The free radicals formed by reaction with H_2O_2 or LOOH and Fe^{2+} were detected by spin trapping methods with PBN (9). Two *N*-oxyl-4,4′-dimethyloxazolidine derivatives of stearic acid, 5-NS and 16-NS, were used as spin labels to probe the hydrophobic interior of the TTAB micelles at different depths. The PBN and NS labeled micelles were prepared as follows: a solution of 10 μmol of fatty acid and 10 or 25 μmol of PBN or 50 nmol of 5-NS or 16-NS in chloroform was placed in a test tube, and the solvent was evaporated under nitrogen. The resulting film of PBN or NS was dispersed in a SDS or TTAB solution. The reaction was started by the addition of $FeSO_4$ to the micellar solution. The mixture was then transferred to a flat rectangular ESR cell, and the ESR spectra of PBN-adducts were recorded under the following conditions: microwave power, 8 mW; magnetic field, 3377 G; and scan range, 50 G. Hyperfine splitting parameters and *g* values were determined by direct comparison with a known standard, Fremy's salt. The ESR spectra of 5-NS and 16-NS were recorded under the following conditions: microwave power, 8 mW; magnetic field, 3374 G; scan range, 25 G; and modulation width, 1.0 G. The concentrations of spin probes in the TTAB micelles were determined from their ESR signals using a standard calibration chart.

RESULTS

The effects of H_2O_2, LOOH, Fe^{2+} and NTA on the peroxidation of LA were studied in TTAB and SDS micelles (Fig. 1). When LA without LOOH was solubilized in positively charged TTAB micelles, lipid peroxidation was strongly induced by H_2O_2 and Fe^{2+} (H_2O_2 dependent lipid peroxidation). This peroxidation was inhibited 30% and 13% by 50 mM mannitol and *t*-butanol, respectively. In negatively charged SDS micelles, however, H_2O_2 dependent lipid peroxidation was not induced. Rather the addition of free Fe^{2+} resulted in the peroxidation of LA in the presence of LOOH instead of H_2O_2 in SDS micelles (LOOH dependent lipid peroxidation), but not in TTAB micelles. LOOH dependent lipid peroxidation in TTAB micelles was induced by Fe^{2+} in the presence of the negatively charged iron chelator NTA.

Figure 2 shows the oxidation of Fe^{2+} associated with H_2O_2 and LOOH dependent peroxidation of LA. In TTAB micelles, Fe^{2+} was oxidized concurrently with

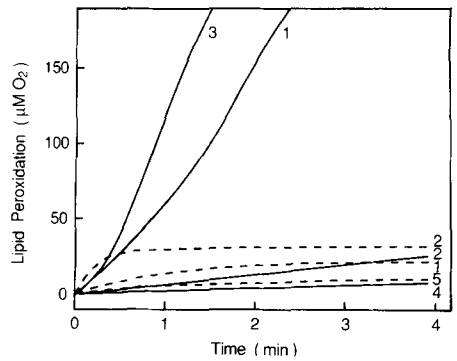

FIG. 1. Peroxide-dependent peroxidation of LA in TTAB micelles (—) at pH 3.5 and in SDS micelles (——) at pH 5.0 (1) H_2O_2, LA and Fe^{2+}, (2) LOOH, LA and Fe^{2+}, (3) LOOH, LA, NTA and Fe^{2+}, (4) LA, NTA and Fe^{2+}, and (5) LA and Fe^{2+}. The LA (25 μmol) was pretreated with 0.5 μmol TPP to remove contaminating LOOH (0.31 μmol). The concentrations of reactants were 5 mM LA, 62 μM LOOH, 100 μM H_2O_2, 30 μM $FeSO_4$, 30 μM NTA, 100 μM TPP, and 50 mM SDS or TTAB. Reactions were started by the addition of Fe^{2+} to the reaction mixtures at 25°C. The amounts of LOOH formed during the 2-min incubation were 143.5 μM (line 1), 47.5 μM (line 2), 388.4 μM (line 3), and 5.4 μM (line 4) in TTAB micelles, and 7.5 μM (line 1) and 20.5 μM (line 2) in SDS micelles.

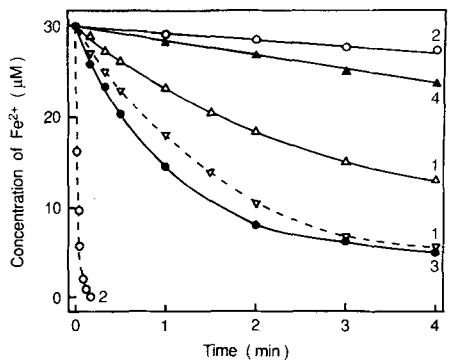

FIG. 2. Oxidation of Fe^{2+} during peroxidation of LA in TTAB micelles (—) and SDS micelles (——). (1) H_2O_2, LA and Fe^{2+}, (2) LOOH, LA and Fe^{2+}, (3) LOOH, LA, NTA and Fe^{2+}, and (4) LA, NTA and Fe^{2+}. The concentrations of reactants and reaction conditions were the same as in Figure 1.

H_2O_2 dependent lipid peroxidation. However, in SDS micelles, Fe^{2+} was also oxidized by H_2O_2 although there was no H_2O_2 dependent lipid peroxidation. During LOOH dependent lipid peroxidation in SDS micelles, free Fe^{2+} was rapidly oxidized in the absence of NTA. However, NTA was necessary in TTAB micelles for the oxidation of Fe^{2+} associated with the LOOH dependent peroxidation of LA.

Figure 3 shows the ESR spectra of PBN adducts formed in the presence of H_2O_2 and LOOH in SDS and TTAB micelles. In SDS micelles, an ESR spectrum attributed to PBN-OH• adducts was observed, while peroxidation of LA was not induced by the addition of H_2O_2 and Fe^{2+}. However, there was little production of PBN-OH• adducts in TTAB micelles while H_2O_2 dependent lipid peroxidation was initiated. With LOOH, a strong ESR spectrum of PBN adduct trapping of alkoxy radical (PBN-LO•) was observed upon addition of free Fe^{2+} to SDS micelles, but was barely seen in TTAB micelles. However in TTAB micelles, an ESR spectrum of PBN-LO• adducts appeared after the addition of Fe^{2+} in the presence of NTA and LOOH.

Nitroxide radicals (NS) such as 5-NS and 16-NS labeled at different positions in liposomal membranes are reported to be lost, possibly by reacting with radicals formed during peroxidation of membrane lipids (17):

$$NS + LO\cdot \text{ or } LOO\cdot \rightarrow \text{Non-radical products} \quad [3]$$

Therefore, to determine the sites at which LO• and LOO• are generated during LOOH dependent lipid peroxidation induced by Fe^{2+} and NTA in TTAB micelles, we examined the consumption of their spin probe radicals. As shown in Table 1, the amount of 16-NS consumed was much greater than that of 5-NS during LOOH dependent lipid peroxidation. Furthermore, the rate and extent of loss of 16-NS by the LOOH dependent Fenton-like reaction were higher in micelles containing LA than in micelles containing lauric acid (LauA). There was little difference in the rate and extent of loss of 5-NS in LA micelles and LauA micelles.

As shown in Figure 4, addition of NTA and Fe^{2+} induced rapid peroxidation of LA in TTAB micelles in the

TABLE 1

Decrease of ESR Signals of 16-NS and 5-NS Induced by Reaction of LOOH with NTA-Fe^{2+} and Inhibition by α-Tocopherol[a]

	% Remaining NS		% Inhibition by α-Toc[b]	
	16-NS	5-NS	16-NS	5-NS
LA	42.7 ± 5.3	64.7 ± 5.8	67.7	21.9
LauA	64.4 ± 3.6	64.9 ± 3.9		

[a] The concentrations of reactants were 50 μM 5-NS or 16-NS, 10 mM LA or LauA, 2 mM LOOH, 0.2 mM NTA, 0.2 mM $FeSO_4$, and 100 mM TTAB. The incubation time was 2.5 min. Other reaction conditions were the same as in Figure 1.

[b] Inhibitions are shown as decreases of ESR signals of 16-NS and 5-NS in the presence of 50 μM α-tocopherol (as percentages of those in its absence).

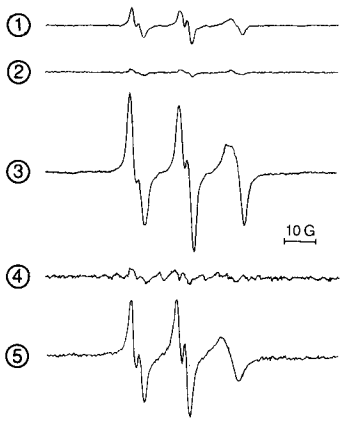

FIG. 3. ESR spectra of the PBN spin adducts. (1) H_2O_2 and Fe^{2+} in SDS micelles, (2) H_2O_2 and Fe^{2+} in TTAB micelles, (3) LOOH and Fe^{2+} in SDS micelles, (4) LOOH and Fe^{2+} in TTAB micelles, and (5) LOOH, NTA and Fe^{2+} in TTAB micelles. Spectra were recorded 30 min after addition of Fe^{2+} at room temperature. The concentrations of reactants in the H_2O_2 system were 25 mM PBN, 2 mM H_2O_2, 0.2 mM Fe^{2+} and 50 mM SDS or TTAB. ESR parameters of spectrum (1) (PBN-OH•); A_N = 15.52 G, A_H = 3.04 G, g = 2.00600. Modulation width, 0.5 G. The concentrations of reactants in the LOOH system were 25 mM PBN, 1.7 mM LOOH, 0.2 mM NTA, 0.2 mM Fe^{2+} and 50 mM SDS or TTAB. ESR parameters of spectrum (3) (PBN-OL•); A_N = 14.77 G, A_H = 3.24 G, g = 2.00560. Modulation width, 0.5 G. ESR parameters of spectrum (5) (PBN-OL•); A_N = 15.39 G, A_H = 2.18 G, g = 2.00600. Modulation width, 1.6 G.

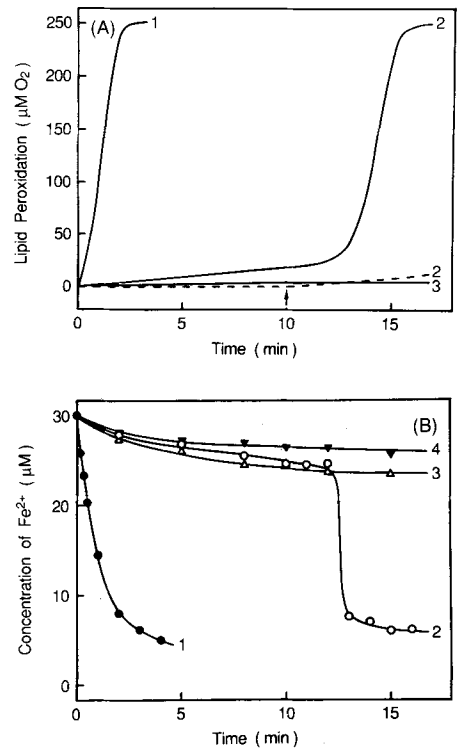

FIG. 4. (A) Peroxidation of LA induced independently of peroxides by chelated Fe^{2+} with NTA, and (B) associated oxidation of Fe^{2+} in TTAB micelles at pH 3.5. (1) LOOH, LA, NTA and Fe^{2+}, (2) LA, NTA and Fe^{2+}, (3) LauA, NTA and Fe^{2+}, and (4) LauA and Fe^{2+}. Reactions were started by addition of Fe^{2+} to the reaction mixtures except for reaction 2 shown by the dotted line, in which Fe^{2+} was added at the time indicated by an arrow. LA (25 μmol) was pretreated with 0.25 μmol TPP. The LOOH concentration was 15.4 μM. The concentrations of other reactants and conditions were the same as in Figures 1 and 2.

presence of LOOH, with associated rapid oxidation of Fe^{2+}. In the absence of LOOH, peroxidation was induced by NTA and Fe^{2+} after a long lag time; the associated oxidation of Fe^{2+} was slow but significant during the lag period and rapid after the lag time. However, the addition of Fe^{2+} after preincubation of TTAB micelles in the presence of NTA for a period corresponding to the lag time did not induce the peroxidation of LA. These results are summarized in Table 2.

α-Tocopherol inhibited H_2O_2 and LOOH dependent lipid peroxidation in TTAB micelles but accelerated LOOH dependent lipid peroxidation in SDS micelles (Fig. 5). In SDS micelles, Fe^{3+} also strongly initiated LOOH dependent lipid peroxidation in the presence of α-tocopherol, but not in the absence of α-tocopherol (data not shown). Figure 6 shows that α-tocopherol was oxidized and free Fe^{3+} was reduced by the interaction of the two in SDS micelles, but α-tocopherol did not react with NTA-Fe^{3+} or free Fe^{3+} in TTAB micelles.

The consumption of 5-NS and 16-NS during LOOH dependent lipid peroxidation in TTAB micelles was also inhibited by α-tocopherol. As shown in Table 1, α-tocopherol inhibited the loss of 16-NS more than of 5-NS.

Figure 7 indicates the time course of oxidation of α-tocopherol during the H_2O_2 and LOOH dependent Fenton reactions in TTAB micelles. The oxidation rates and amounts of α-tocopherol induced by H_2O_2 and Fe^{2+} in LauA and LA micelles were similar, but those induced by LOOH and NTA-Fe^{2+} were greater in LA micelles than in LauA micelles.

Table 3 shows the fluorescence intensities of α-tocopherol in SDS and TTAB micelles and in alcohols of different chain length. The organic solvents affected the fluorescence intensity, which increased as the chain length of the alcohol increased. The fluorescence intensity of α-tocopherol in TTAB micelles was as strong as that in ethanol containing 1% H_2O, but the fluorescence was only about 30% as much in SDS micelles than it was in TTAB micelles. The fluorescence in TTAB micelles decreased in the presence of the membrane probes such as 5-NS and 16-NS (data not shown), which have been used to determine the location of chromophores in membranes by measuring their quenching efficiencies (18). The Stern-Volmer plots of quenching of α-tocopherol fluorescence were linear; no difference in quenching was observed between 5-NS and 16-NS (9).

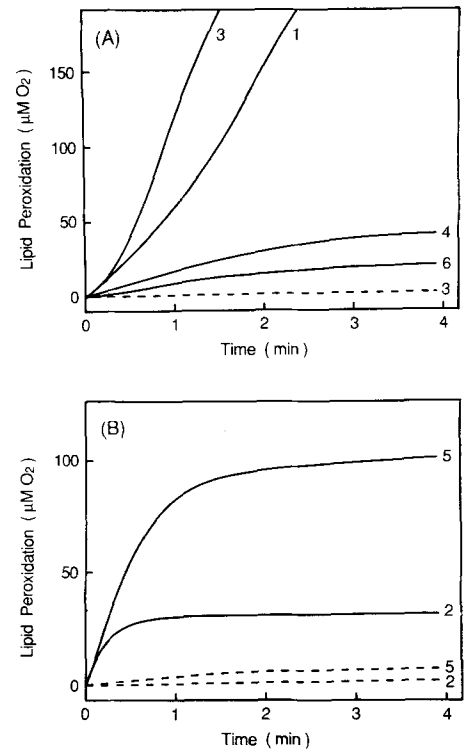

FIG. 5. Effect of α-tocopherol on the H_2O_2 and LOOH dependent peroxidation of LA in (A) TTAB micelles and in (B) SDS micelles. (1) H_2O_2, Fe^{2+} and LA, (2) LOOH, Fe^{2+} and LA or LauA, (3) LOOH, Fe^{2+}, NTA and LA, (4) H_2O_2, Fe^{2+}, α-Toc and LA, (5) LOOH, Fe^{2+}, α-Toc and LA or LauA, and (6) LOOH, Fe^{2+}, NTA, α-Toc, and LA. Solid lines and dotted lines show the systems containing LA and LauA, respectively. The concentration of α-Toc was 100 μM. The concentrations of other reactants were the same as in Figure 1.

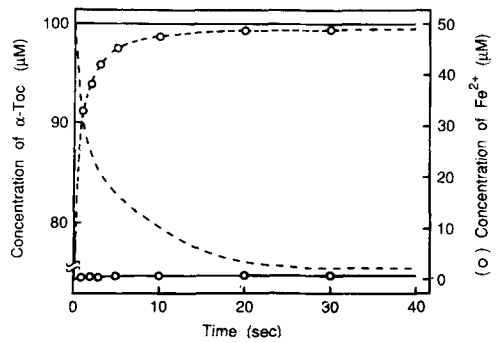

FIG. 6. Time courses of oxidation of α-tocopherol and reduction of free Fe^{3+} or Fe^{3+} chelated with NTA by their interaction in TTAB micelles (—) with and without NTA and in SDS micelles (——) without NTA. The concentrations of reactants were 100 μM α-Toc, 50 μM $FeNH_4(SO_4)_2$, 50 μM NTA and 50 mM TTAB or SDS.

DISCUSSION

In TTAB micelles, the peroxidation of LA was induced after addition of H_2O_2 and Fe^{2+}, but PBN-OH• adducts

TABLE 2

Peroxide Dependent and Independent Initiation of Lipid Peroxidation, and Formation of Initiators and Their PBN-Adducts by Fe^{2+} and NTA-Fe^{2+} in SDS and TTAB Micelles

Micelle system	Initiation of lipid peroxidation			PBN adduct formation		Formation of initiator (oxidation rate of Fe^{2+}; μM/min)		
	H_2O_2 dependent	LOOH dependent	Peroxide independent	H_2O_2 system	LOOH system	H_2O_2 system	LOOH system	System without peroxide[a]
SDS (Fe^{2+})	—	+	NE[b]	+	+	17.0	829	<0.2
TTAB (Fe^{2+})	+	—	—	—	—	9.0	0.8	0
TTAB (Fe^{2+},NTA)	NE[b]	+	+	NE[b]	+	NE[b]	25.0	1.2

[a]Autoxidation. [b]NE, not examined.

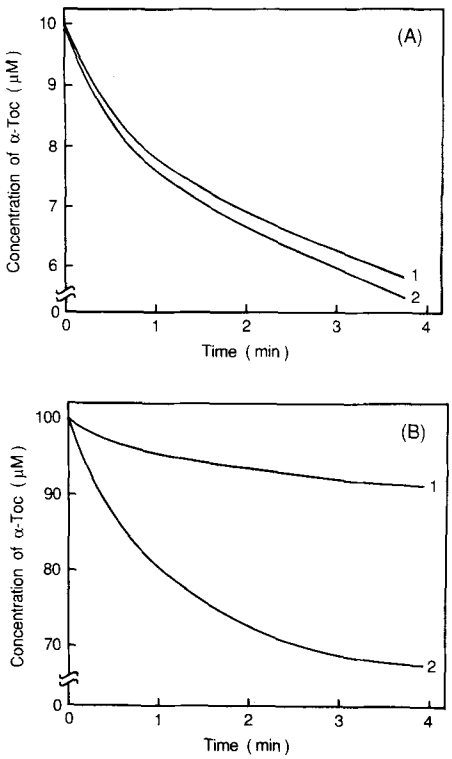

FIG. 7. Oxidation of α-tocopherol in (A) H_2O_2 and (B) LOOH systems in TTAB micelles. (1) LauA, and (2) LA. The concentrations of reactants were (A) 10 μM α-Toc, 0.1 mM H_2O_2, 5 mM fatty acid and 10 μM $FeSO_4$, and (B) 100 μM α-Toc, 100 μM LOOH, 5 mM fatty acid, 50 μM NTA and 50 μM $FeSO_4$.

were not formed (Figs. 1 and 3). The OH radical formed by the reaction of H_2O_2 and Fe^{2+} [1] (Fig. 2) is probably responsible for the initiation [eq. 4] of peroxidation of LA. The processes are then propagated by a chain reaction [eqs. 5 and 6] in TTAB micelles; the processes are inhibited by OH-radical scavengers.

$$LH + \cdot OH \rightarrow L\cdot + H_2O \quad [4]$$

$$L\cdot + O_2 \rightarrow LOO\cdot \quad [5]$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot \quad [6]$$

In positively charged TTAB micelles, Fe^{2+} is present in the inner water phase and the outer bulk water phase, but not at the positively charged surface. Therefore, some of the ·OH formed in the inner hydrophobic region may react with the unsaturated moiety of LA in the micelles resulting in the initiation of lipid peroxidation. However, since a radical trapping site of PBN is present at the surface, it cannot interact with ·OH. At the surface, ·OH is not formed because no Fe^{2+} is present.

By contrast, PBN-OH· adducts were observed in SDS micelles, but H_2O_2 dependent lipid peroxidation was not induced (Figs. 1 and 3). Because positively charged Fe^{2+} is present at the surface of negatively charged SDS micelles resulting from ionic interaction with the sulfate group of SDS, ·OH is also formed at the surface. Therefore, ·OH can react with the radical trapping site of PBN at the surface, but cannot react with the unsaturated moiety of LA located in the inner hydrophobic region.

The LOOH dependent initiation of lipid peroxidation by free Fe^{2+} and the concurrent oxidation of Fe^{2+} were observed in negatively charged SDS micelles but not in positively charged TTAB micelles (Figs. 1 and 2). Fridovich (19) suggested that polyanionic plasma membranes contain some bound Fe^{2+}, and that ROOH dependent lipid peroxidation can occur when traces of lipid hydroperoxide are present. In SDS micelles, the positively charged Fe^{2+} located at the negatively charged micelle surface plays a role in the initial breakdown of LOOH, which may also be present on the surface, to form LO· [eq. 7] for the chain reaction [eqs. 8, 5 and then 6].

$$LOOH + Fe^{2+} \rightarrow LO\cdot + Fe^{3+} + OH^- \quad [7]$$

$$LH + LO\cdot \rightarrow L\cdot + LOH \quad [8]$$

In contrast, Fe^{2+} may not be able to react with LOOH in TTAB micelles because Fe^{2+} is not located in the positively charged surface of the membranes, and consequently LOOH dependent peroxidation of LA is not initiated. These conclusions are supported by the finding that an ESR spectrum of PBN-LO· adducts was observed in SDS micelles but not in TTAB micelles after the addition of Fe^{2+} to LOOH (Fig. 3). In the presence of NTA, however, LOOH dependent peroxidation of LA and

TABLE 3

Relative Fluorescence Intensities and UV Absorbances of α-Tocopherol in Different Solvents[a]

Solvent system	Fluorescence			UV absorption	
	Relative intensity[b]	Excitation maximum (nm)	Emission maximum (nm)	Absorbance	Absorption maximum (nm)
SDS micelles	30.9	295	326	0.325	293
TTAB micelles	100	295	326	0.325	295
Ethanol	184.8	295	326	0.326	292
Ethanol (1% H_2O)	90.8	295	330	0.326	292
Butanol	214.8	295	328	0.325	292
Octanol	271.9	295	324	0.325	292

[a]A solution of 0.5 μmol α-tocopherol in chloroform was evaporated under nitrogen, and the residue was dissolved in 5 mL of organic solvents or dispersed in 5 mL of 50 mM SDS or TTAB solution with a vortex mixer and sonicator.

[b]Relative fluorescence intensities of α-tocopherol were calculated as percentages of that in TTAB micelles.

PBN-LO· adduct formation were catalyzed by Fe^{2+} in TTAB micelles with concurrent oxidation of Fe^{2+} (Figs. 1–3). Fe^{2+} probably binds to negatively charged NTA located at the positively charged surface of TTAB micelles and then reacts with the OOH group of LOOH at the membrane surface.

The site of the propagation reaction of LOOH dependent oxidation of LA catalyzed by NTA and Fe^{2+} in TTAB micelles was investigated by following the consumption of the spin probes 5-NS and 16-NS (Table 1). More 16-NS than 5-NS was consumed during LOOH dependent peroxidation, indicating that more radicals are present in the deeper regions of the micelles. The consumption of 16-NS during the LOOH dependent NTA-Fe^{2+}-catalyzed Fenton-like reaction [eq. 7] was greater in LA micelles than in LauA micelles. This difference may be due to a LOO· because 16-NS may react with LO· and LOO· in LA micelles but only with LO· in LauA micelles. Decreases in 5-NS during the LOOH dependent Fenton-like reaction were similar in LA and LauA micelles indicating that 5-NS reacts mainly with LO·. These results suggest that the LO· formed at the membrane surface from LOOH [eq. 7] penetrates into the hydrophobic region and abstracts the methylene hydrogen from LA [8]. The propagation reaction [eq. 6] may take place in the deeper regions of the membrane because the LOO· regenerated reacted mainly with 16-NS.

In TTAB micelles in the absence of LOOH, NTA-Fe^{2+} induced peroxidation of LA after a lag period. A small amount of LOOH was probably formed independently of preformed peroxides by NTA-Fe^{2+}, and then rapid peroxidation of LA occurred dependent upon the LOOH accumulated during the lag period associated with the rapid oxidation of Fe^{2+}. The addition of Fe^{2+} after preincubation did not induce peroxidation of LA. The amount of LOOH formed without NTA and Fe^{2+} during preincubation with LA might not have been sufficient to catalyze the LOOH dependent peroxidation of LA. The autoxidation rate of Fe^{2+} was significantly increased by iron chelators such as NTA and diethylenetriaminepentaacetic acid with concomitant O_2 uptake in the absence of LA (data not shown). Therefore, a ferric-oxygen complex may be the initiating species of peroxide-independent lipid peroxidation. However, this remains to be established.

It is uncertain whether the chromanol group of α-tocopherol in membranes is located at the surface (17,20) or in the inner hydrophobic region (21,22). The fluorescence intensity of α-tocopherol decreased as the polarity of the solvent increased (Table 3). The much lower fluorescence intensity of α-tocopherol in SDS micelles than in TTAB micelles (Table 3) indicated that the chromanol group is present in a more polar environment in SDS micelles than in TTAB micelles. Since α-tocopherol was rapidly oxidized by Fe^{3+} in SDS micelles, a phenolic hydroxyl group of α-tocopherol may be associated with the SO_3^- group of SDS through hydrogen bonding and exposed at the membrane surface. In TTAB micelles, however, the membrane probe 16-NS quenched intrinsic α-tocopherol fluorescence as effectively as 5-NS did. This suggests that the chromanol moiety of α-tocopherol is uniformly distributed in the shallow and the deep hydrophobic regions of TTAB micelles.

α-Tocopherol inhibited H_2O_2 dependent and LOOH dependent peroxidation of LA catalyzed by free and chelated Fe^{2+}, respectively, in TTAB micelles, but enhanced free Fe^{2+} induced LOOH dependent lipid peroxidation (Fig. 5). The enhancing effect of α-tocopherol in SDS micelles may be due to its regeneration of Fe^{2+} from Fe^{3+} [eq. 9],

$$\alpha\text{-Toc} + Fe^{3+} \rightarrow \alpha\text{-Toc}\cdot + Fe^{2+} \qquad [9]$$

because i) α-tocopherol rapidly reduced Fe^{3+} to Fe^{2+} in SDS micelles, but not in TTAB micelles (Fig. 6), ii) α-tocopherol caused Fe^{3+} to induce LOOH dependent peroxidation of LA in SDS micelles, whereas this peroxidation was not induced by Fe^{3+} in the absence of α-tocopherol, and iii) the fluorescence intensity of α-tocopherol was much lower in SDS micelles than in TTAB micelles (Table 3).

No significant difference was observed in the rates of oxidation of α-tocopherol by H_2O_2 and Fe^{2+} in TTAB micelles containing unsaturated LA or saturated LauA (Fig. 7A). These results indicate that in this system α-tocopherol inhibits the H_2O_2 dependent peroxidation of LA by scavenging ·OH [eq. 10] rather than free lipid radicals [eqs. 11,12].

$$\cdot OH + \alpha\text{-Toc} \rightarrow \alpha\text{-Toc}\cdot + H_2O \qquad [10]$$

$$LOO\cdot + \alpha\text{-Toc} \rightarrow \alpha\text{-Toc}\cdot + LOOH \qquad [11]$$

$$LO\cdot + \alpha\text{-Toc} \rightarrow \alpha\text{-Toc}\cdot + LOH \qquad [12]$$

By contrast, as shown in Figure 7B, much more α-tocopherol was oxidized during the NTA-Fe^{2+} catalyzed LOOH dependent Fenton reaction [eq. 7] in TTAB micelles containing LA, in which LO· and LOO· are generated [eq. 7, 5], than in TTAB micelles containing LauA, in which only LO· is produced [eq. 7]. These findings suggest that α-tocopherol inhibits the LOOH dependent peroxidation of LA catalyzed by NTA-Fe^{2+} in TTAB micelles mainly by scavenging LOO· [11] in the deep inner hydrophobic membrane regions, where 16-NS was almost completely consumed by LOO· (Table 2). In the presence of α-tocopherol, the LO· initially formed by a Fenton-like reaction [eq. 7] may be lost in the following three reactions: i) interaction of LO· [13], ii) reaction with α-tocopherol [12], and iii) reaction with LA to form LOO· [8, 5], which is also reduced by α-tocopherol [11]. Since the concentration of LA is much higher than the concentrations of LOOH and α-tocopherol, reactions [8] and [11] may progress preferentially to reactions [12] and [13].

$$LO\cdot + LO\cdot \rightarrow \text{Stable product} \qquad [13]$$

Our results and conclusions on the effects of α-tocopherol on H_2O_2 and LOOH dependent peroxidation of LA in SDS and TTAB micelles are summarized in Table 4.

ACKNOWLEDGMENTS

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TABLE 4

Effects of α-Tocopherol on H_2O_2 and LOOH Dependent Lipid Peroxidation

Initiation system	Lipid peroxidation	Reaction species	Reaction site of α-Toc
H_2O_2, Fe^{2+} (TTAB)	Decrease	•OH	Unsaturated bonding moiety
LOOH, Fe^{2+} (SDS)	Increase	Fe^{3+}	Membrane surface
LOOH, NTA-Fe^{2+} (TTAB)	Decrease	LOO•>LO•	LOO• at 16-NS LO• at 5-NS and 16-NS

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The Inhibitory Effect of Water on the Co^{2+} and Cu^{2+} Catalyzed Decomposition of Methyl Linoleate Hydroperoxides[1]

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The inhibitory effect of water on the decomposition of methyl linoleate hydroperoxides (MLHP) catalyzed by Co^{2+} and Cu^{2+} was studied in a model system using proton nuclear magnetic resonance (NMR) spectroscopy. MLHP were prepared by photoxidation and purified by chromatographic methods. Proton NMR spectroscopy was used to measure reaction rates by monitoring changes in the intensity of the OOH signal. The rate constant of the reaction was obtained by plotting the natural logarithm of MLHP concentration *vs* time. In the first part of the study, no transition metals were added to the model system, so that the effect of water could be attributed to the interaction between water and MLHP only. The rate constant of the reaction (K) was found inversely proportional to the concentration of water. There was a downfield chemical shift of both hydroperoxide and water peaks in the NMR spectra when water was added. As temperature increased to 40°C, the difference in K between the systems with 0% and 2% water disappeared. It is proposed that the hydroperoxides were solvated with water which retarded their decomposition. When Co^{2+} was added to the model system, K decreased as the concentration of water increased from 0% to 1.5%. As temperature increased from 18°C to 40°C, differences between the K for 0% and 2% water disappeared. A similar phenomenon was observed in reactions catalyzed with Cu^{2+}. These findings would support a mechanism in which the protective effect of water involves both the solvation of OOH and hydration of the metal catalyst.
Lipids 27, 234-239 (1992).

Lipid autoxidation, a free radical chain reaction, is a recognized problem in preserving food stuffs and involves unsaturated lipids reacting with oxygen. The mechanism of lipid autoxidation has been studied by several research groups and reviewed (1-4). The overall reaction consists of four major stages: initiation, propagation, chain branching and termination.

Decomposition of lipid peroxides plays an important role in autoxidation of unsaturated lipids. First, during the propagation stage, hydroperoxide decomposition occurs with alkoxy and/or peroxyl radicals as reaction products being formed. These radicals in turn abstract hydrogens from fatty acids to form more hydroperoxides and perpetuate the chain reaction. Chain propagation accelerates autoxidation because the reactions of alkoxy and peroxyl radicals with lipid molecules are faster than *ab initio* formation of free radicals by light, heat, or other energy sources. Second, the volatile breakdown products from the reaction are directly responsible for undesirable flavors in rancid foods. The autoxidation of lipids and the generation, synthesis and decomposition of lipid peroxides have been studied extensively during the past two decades (5-17).

Transition metals, such as iron, copper and cobalt, are found in many foods and are important elements in the decomposition of lipid peroxides. Because of their unpaired electron(s) in 3d or 4s orbital, transition metals readily lose or gain an electron so they serve as excellent catalysts of the reaction. The role of transition metals in the decomposition of lipid peroxides has been investigated and reviewed (18-22).

Water retards lipid autoxidation in many dehydrated and low moisture foods (23,24). The shelf life of oat flakes containing 2-6% water was less than two weeks compared with eight months for flakes with 10% moisture (25). Several hypotheses have been offered to explain this inhibitory effect (26-28). The presence of water could slow autoxidation by the following mechanisms: (a) lowering diffusion rate of oxygen; (b) lowering effectiveness of metal catalysts; (c) promoting non-enzymatic browning which produces antioxidants; and (d) excluding air from the surface of food. Because of the complexity of food systems, however, the effect of water observed in the studies cited above does not support or rule out any single one of these possibilities.

The effect of water on lipid autoxidation has been investigated in model systems using methyl linoleate as substrate (29-32). Based on systematic studies, Karel (33) suggested the following events occurred during autoxidation which retarded degradation of lipid peroxides: (a) hydrogen-bonding of amphipolar hydroperoxides at the lipid-water interface lowered the total effective hydroperoxide concentration; and (b) hydration of metal reduced the effectiveness of the catalysts.

This study was undertaken to provide data to validate earlier hypotheses and to increase our understanding of how water interacts with hydroperoxides and transition metals to retard lipid autoxidation. A new model system was developed that contained methyl linoleate hydroperoxides (MLHP), transition metals and various amounts of water in fully deuterated acetone (acetone-d_6). Proton nuclear magnetic resonance (NMR) was used to monitor the reaction by measuring the area of the OOH peak. The area of this peak is proportional to the concentration of MLHP in the system, and thus changes as decomposition occurring can be measured. The inhibitory effect of water was investigated by: (a) studying the interaction between water and lipid peroxides; and (b) investigating the interaction between water and transition metals.

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Abbreviations: acetone-d_6, fully deuterated acetone; HPLC, high-performance liquid chromatography; K, apparent rate constant; MLHP, methyl linoleate hydroperoxides; NMR, nuclear magnetic resonance; r^2, linear regression correlation coefficient; UV, ultraviolet.

MATERIALS AND METHODS

Materials. The following chemicals were used to prepare and purify MLHP: methyl linoleate (NuChek Prep, Elysian, MN); rose bengal (cert.) (Eastman Kodak, Rochester, NY); silicic acid (n-hydrate, J.T. Baker Chemical Co., Phillipsburg, NJ), hexane and methanol. Fully deuterated acetone (acetone-d_6) (Wilmand Glass Co., Buena, NJ) was used as solvent in the model system. Cobalt(II)chloride, $CoCl_2 \cdot 6H_2O$ (crystal) and Cu(II)chloride, $CuCl_2 \cdot 2H_2O$ (crystal) (J.T. Baker) were used as catalysts.

Preparation of MLHP. The photoxidation method reported by Schenk and Schults-Elte (34) was modified slightly to synthesize MLHP. Three grams of methyl linoleate, 0.05 g rose bengal and 70 mL of methanol were mixed in a 150-mL Erlenmeyer flask. The mixture was transferred to a 100-mL fritted glass funnel connected to an oxygen tank. Two 300-W spotlights were used as an energy source for the photochemical reaction. Temperature was maintained at 2°C using a refrigerated water bath covered with a glass baking dish containing water to act as an infrared light filter. Water was added to compensate for evaporation during the 18-hr reaction. When the reaction was stopped, the methanol was removed under vacuum in a rotary evaporator.

Purification of MLHP was done by column chromatography (35). Silicic acid (70 g) was activated in a 110°C oven for 12 hr, cooled in a desiccator, transferred to a 500-mL beaker and mixed with enough hexane to form a slurry. The slurry was packed into a 400 mm × 25 mm glass column and washed with 500 mL of hexane. Products from the photochemical reaction were loaded onto the column in a minimal volume of hexane/diethyl ether (15:5, v/v). Five hundred milliliters of hexane/diethyl ether (95:5, v/v) were used to elute non-polar compounds and unreacted methyl linoleate. MLHP were eluted with 500 mL of hexane/diethyl ether (80:20, v/v). A drop-controlled fractionator was used to collect MLHP in disposable culture tubes. Thin-layer chromatography served to identify tubes containing MLHP (36). ^1H NMR spectrometry was employed to confirm the presence of MLHP in combined fractions. High-performance liquid chromatography (HPLC) was used to further purify MLHP. Ten microliters of the MLHP mixture were loaded onto a silica gel semipreparative column (10 mm × 300 mm). The elution solvent was 1.2% isopropanol and 98.8% hexane (v/v). The flow rate of the solvent was 4 mL/min. The wavelength of the ultraviolet (UV) detector was set at 245 nm. Solvent in the collected MLHP fraction was evaporated with a rotary evaporator. The purified MLHP was transferred to small glass vessels, flushed with nitrogen, and stored at −22°C.

Preparation of model system. The model system was prepared in an NMR tube using 50 μL of acetone-d_6 as solvent. To investigate the interactions between MLHP and water, the solvent was replaced with various amounts of distilled water (v/v) to arrive at a final concentration of water of 0%, 0.5%, 1.0%, 1.5%, or 2.0%. Interactions between transition metals and water were studied by replacing a like volume of acetone-d_6 in the system with a solution of cobalt(II)chloride or copper(II)chloride dissolved in acetone-d_6.

Experimental procedure. The proton NMR spectrum of MLHP shown in Figure 1 was recorded on a JEOL FX90Q FT-NMR spectrometer (Tokyo, Japan) operating at 89.90 MHz. Complete identification and interpretation of the spectra of methyl linoleate and MLHP have been previously reported by Gunstone and Norris (37), Chan (3), Chan and Levette (15), and Frankel *et al.* (8). The OOH peak appeared downfield at 10.40 ppm. The peak at 3.65 ppm can be assigned to OCH_3, and was chosen as a reference peak for measuring OOH peak areas. The area for OOH protons was integrated using the operating disk FAFT72 in the JEOL computer. Since the initial concentrations of OOH were known, the areas of OOH at any given time could be converted into molar concentrations. For each data point, three readings were taken. The average standard deviation was 0.011. All measurements were conducted at 18°C unless specified otherwise.

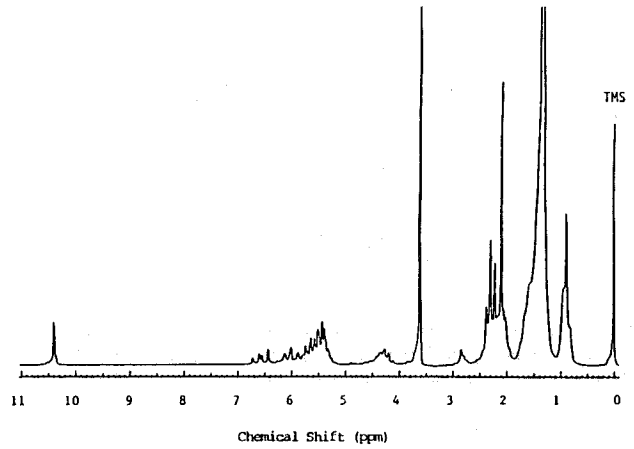

FIG. 1. Proton NMR spectrum of MLHP (89.90 MHz). Peak of OOH appeared at 10.40 ppm. Peak at 3.65 ppm was assigned to OCH_3 and was used as reference for the measurement of the peak area of OOH.

To determine the apparent rate constant for the reaction, K, the natural logarithm of the concentration of MLHP was plotted against time. The slope of the straight line is K. All statistical analyses, such as linear regression correlation coefficient (r^2) and confidence level, were carried out using the statistical program, MSUSTAT, on an IBM computer.

RESULTS AND DISCUSSION

For convenience of discussion, our results are presented in two parts: (i) solvation of MLHP; and (ii) hydration of metals. In the first part, transition metals were not involved so that the effect of water could be attributed to the solvation of MLHP only. In the second part, water, MLHP and metals were present in the same system; so the effect of water could be attributed to solvation of MLHP and to hydration of metals.

Solvation of MLHP. The apparent rate constant, K, for the decomposition of MLHP, as affected by various concentrations of water, is shown in Figure 2. K decreased linearly as the concentration of water increased, indicating that water acts as an inhibitor of the reaction.

A downfield shift of the OOH and water signals in the NMR spectra was observed when water was added to MLHP. The extent of the shift was dependent on the concentration of water in the system (Fig. 3 and Table 1). The

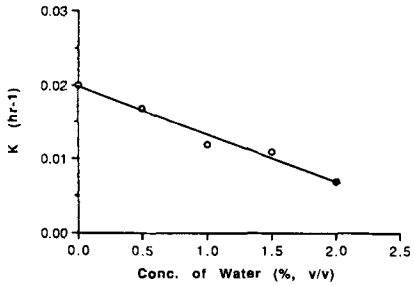

FIG. 2. Plot of apparent rate constant (K) *vs* concentration of water in MLHP decomposition in the absence of metals. Duplicate experiments were carried out ($r^2 = 0.94$, confidence level >99%).

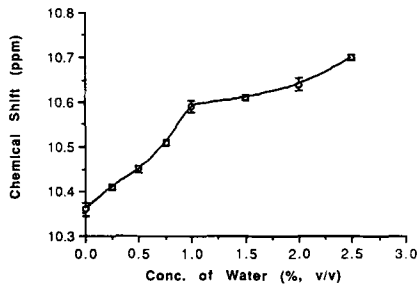

FIG. 4. Change in chemical shift of NMR peak of OOH *vs* increased concentration of water. Standard deviations of the measurements are shown by the error bar.

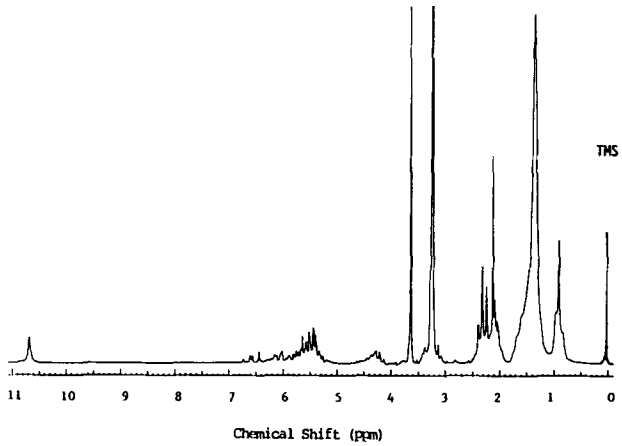

FIG. 3. Proton NMR spectrum of MLHP (89.90 MHz). The model system contained 2% H_2O. Peak for OOH was shifted from 10.40 ppm to 10.65 ppm (Fig. 1). Peak for water appeared at 3.25 ppm.

TABLE 1

Change in Chemical Shift of NMR Peak of Water After Mixing with MLHP

Conc. of H_2O (%, v/v)	Chemical shift (ppm)[a]		
	Before mixing	After mixing	Change
0.25	2.85	2.90	0.05
0.50	2.90	2.95	0.05
0.75	2.93	3.00	0.07
1.00	2.95	3.10	0.15
1.50	3.00	3.15	0.15
2.00	3.10	3.25	0.15

[a]Standard deviation range of the measurements was ±0.007 to 0.014.

observed change in chemical shift can be explained by interaction between the OOH protons and water molecules. Hydrogen bonding would be expected to alter shielding of these protons which would result in the observed downfield shifts. This explanation is supported by the nonlinear change in chemical shift with increasing amounts of water in the system (Fig. 4), in contrast to the linear change that would be expected if replacement of acetone-d_6 was the cause. When the chemical shift was plotted against water concentration (Fig. 4), the curve obtained was somewhat bimodal. Water concentration above 1.0% caused less change per unit increase than at concentrations below 1.0%. This finding supports the mechanism proposed by Karel (4) and by Barclay and Ingold (5) that polar hydroperoxides form water-peroxide complexes which alter the path of the reaction.

This would be expected if hydrogen bonding occurred between the peroxide group and two molecules of water. The molar concentration corresponding to 1% water in the system is approximately twice that of MLHP in the system. The addition of more water would not change the equilibrium of the reaction toward the bound state as rapidly as when water concentrations were lower and more binding sites on the hydroperoxide molecules were free. The preferential bonding between water and one of the isomers in the mixture could also account for the observed non-linear response to increased water concentrations. However, the fact that the oxidation rate and response to water of the 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate isomer, separated by using the method by Chan and Levett (15), was nearly identical to that of the MLHP mixture does not support this hypothesis. The chemical shift of the NMR peak for water was observed to revert back toward initial values as the reaction progressed and the concentration of hydroperoxide decreased (Table 2). The formation and subsequent decay of water-OOH hydrogen

TABLE 2

NMR Chemical Shifts of Water and Hydroperoxide Peaks in Various Systems

System	Chemical shift (ppm)[a]	
	OOH	H_2O
Acetone-d_6 + 2% H_2O		3.15
Acetone-d_6 + 2% H_2O + Co^{2+} (100 ppm)		3.85
Acetone-d_6 + Co^{2+} (100 ppm) + MLHP	10.4	
Acetone-d_6 + MLHP	10.4	
Acetone-d_6 + 2% H_2O + MLHP	10.6	3.20
Acetone-d_6 + 2% H_2O + MLHP + Co^{2+} (100 ppm)	10.6	3.40

[a]Standard deviation range of the measurements was ±0.007 to 0.014.

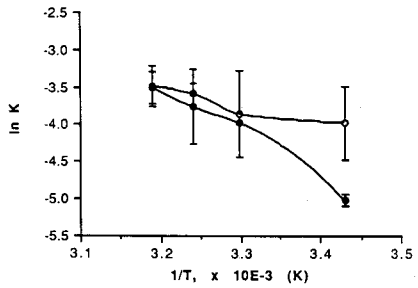

FIG. 5. Plot of K *vs* temperature for reactions in the absence of metals: ○, 0% H_2O; ●, 2% H_2O. Standard deviation of the measurements are shown by the error bar.

bonds could explain this observation. The results support the proposals of Karel (4) and Barclay and Ingold (5) that polar hydroperoxides form water-peroxide complexes which slow their decomposition.

To further support our findings, the effect of temperature on the reaction with 0% and 2.0% water was investigated. At 18°C, the apparent rate constant (K) for MLHP decomposition in the absence of water was twice that of the one with 2.0% water (Fig. 5). The reaction rate of the system with water was more temperature-dependent than the one without water, and the difference in K disappeared at 30°C and the plotted lines met at 40°C.

A possible explanation of this is that at 18°C most of the peroxide moieties were complexed with water, as indicated by the chemical shifts of the water and peroxide peaks. As temperature increased, the hydrogen bonds were weakened and the equilibrium shifted toward more free water and more free peroxide moieties. This would explain the near-linear temperature response of the 2% water system. Unfortunately, neither the NMR signals for water at different temperatures, nor the effect of temperature at lower water concentrations have been determined so far; so this explanation remains tentative. The influence of temperature on hydrogen bonding has been studied previously. Luck (38) reported that as temperature increased, hydrogen bonding between water and alcohol decreased; but this observation was made over a much wider temperature range than used in the present study.

Hydration of metals. The effect of water on the reaction catalyzed by Co^{2+} (Fig. 6) was attributed to both solvation and hydration. Adding 0.5% water to the model system reduced K by 55%. Water concentrations above 0.5% had decreasing effects on reaction rates, which were reduced by 67% and 80% by 1.0% and 1.5% water, respectively. No additional inhibitory effect was found when 2.0% water was added to the system. The supposition that hydration of the metal ion reduces the reaction rate is supported by these results. When 100 ppm Co^{2+} was added to a system containing acetone-d_6, 2% water and no MLHP, a chemical shift in the NMR spectrum of the water peak to 3.85 was observed. This indicated the water was complexing with the metal ion. Decomposition of hydroperoxides catalyzed by metal ions as proposed by Chan (3) involves the exchange of electrons; a) $ROOH + M^{(n+1)} \leftrightarrow ROO^* + H^+ + M^{n+}$, and; b) $ROOH + M^{n+} \leftrightarrow RO^* + OH^- + M^{(n+1)}$. Hydration of metals can reduce their effectiveness as catalysts (19). Pokorny (18) reported that transition metal ions, such as Co^{2+}, are normally surrounded by water molecules and can form polymeric micelles. This can inhibit electron transfer from the outer layer of the metal to the oxidizing form of molecule. Increasing amounts of water in the model system could increase the degree of hydration with subsequent reduction in reaction rates. At 1.5% water, the molar concentration of added water is one-half that of the Co^{2+} ion and is the same when maximum inhibition occurs. This supports the formation of a complex containing two cobalt ions per water molecule.

Experiments to determine the effects of temperature on reactions catalyzed by Co^{2+} and Cu^{2+} with 0% and 2.0% water in the system were conducted. Results (Fig. 7) were similar to those obtained with a non-catalyzed system (Fig. 5). At 18°C, there were differences in the rate constants between the systems with and without water. The

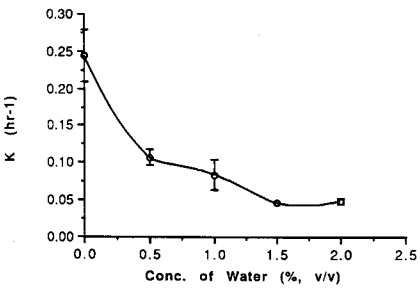

FIG. 6. Change in K *vs* concentrations of water in systems containing 100 ppm Co^{2+}. Standard deviations are shown by error bars.

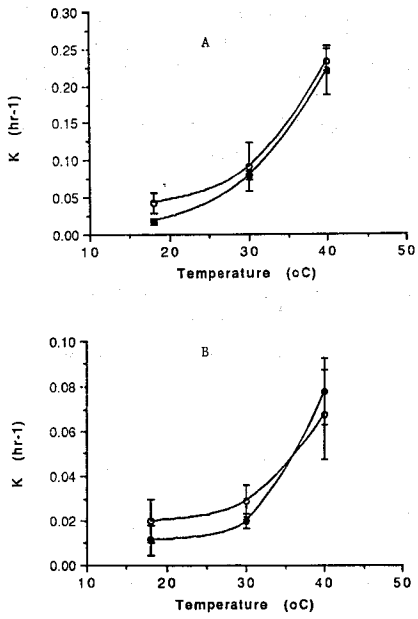

FIG. 7. Plot of K *vs* temperature for reactions catalyzed by Co^{2+} and Cu^{2+}. (A) ○, 0% H_2O and Co^{2+}; ●, 2% H_2O and Co^{2+}. (B) ○, 0% H_2O and Cu^{2+}; ●, 2% H_2O and Cu^{2+}. Concentration of Co^{2+} and Cu^{2+}: 10 ppm. Standard deviation of the measurements are shown by the error bar.

systems with 2.0% water were more temperature-dependent and with both catalysts the K of the reactions increased faster than in systems with no water. Near 30°C, or slightly above, the rates in the Co^{2+} catalyzed reactions were nearly the same for both systems. This effect of temperature on the reactions would be expected if there is an interaction of water and metal which inhibits the reaction at 18°C, but which is disrupted as temperatures increase.

The observed differences between the reactions catalyzed with Co^{2+} and Cu^{2+} could be due to the fact that they catalyze redox reactions by different mechanisms. Copper normally catalyzes *via* direct electron transfer while cobalt more easily forms complexes with polar compounds, such as oxygen and hydroperoxides. The differences in heat of hydration could also account for part of the observed differences. Hydration heat of Cu^{2+} is approximately 11 kcal/mole greater than that for Co^{2+} (39). The data obtained in this study do not enable us to determine which, if either, explanation is correct, but the addition of water inhibited the reactions catalyzed with copper as well as those catalyzed with cobalt.

Transition metals do not exist in the free state but are always surrounded by solvents, such as water, and oxidation products, such as lipid peroxides, to form polymeric micelles (18). The complex formed can stop or slow the electron transfer between the metals and the organic substrate. Hill and McAuley (21) have described how such a complex would interfere with the oxidative decarboxylation of carboxylic acid. Waters (20) indicated that the ease of electron transfer depends on the equilibrium constant for the ligand-displacement process. A MLHP-metal-water complex may actually have been formed in the model system during MLHP degradation.

Indirect evidence for the formation of such a complex is found in the NMR data presented in Tables 2 and 3. The data presented in Table 2 indicate a) that water was bound to Co^{2+} through coordination bonding, which caused a downfield shift of the proton in the presence of water; b) that there was no direct interaction between the peroxide OOH groups and Co^{2+} since the chemical shift of the OOH proton did not change when the two were mixed in the absence of water; and, c) that water interacted with MLHP and Co^{2+} causing a downfield shift of both the protons of water and the OOH groups. Two possible explanations for these findings are; i) Co^{2+} was hydrated to form a water-metal complex and, in an independent reaction, water was bound to OOH groups *via* hydrogen bonding to form a water-hydroperoxide complex. The physical barrier these water complexes create would slow electron transfer from catalyst to substrate and inhibit the reaction; ii) previous studies and our experimental data indicate the possibility that Co^{2+} was bound to water with water molecules surrounding the Co^{2+} ion. Hydrogen bonding of the water molecules with the oxygens of the OOH group of MLHP occurred to form a metal-water-MLHP complex. This is supported by the chemical shift data presented in Table 3. In the reaction with 0.5% water, the chemical shift of the water peak was 2.95 ppm before Co^{2+} was added (0 hr). After mixing (0.25 hr), the peak was downshifted to 3.50 ppm. This is obviously due to a change in the chemical environment of the water protons, which could be caused by water-cobalt complex forming hydrogen bonds with the peroxide OOH group to form a water-MLHP-Co^{2+} complex. As MLHP decomposed, the amount of hydrogen bonding between water and MLHP lessened and the protons in water became more shielded causing the NMR water peak to shift back toward its original position. In our experiments, at the end of the reaction the chemical shift of the water peak was 3.00 ppm. Similar observations were made at other water concentrations. The association between the change in the chemical shift of the water peak and the decomposition of MLHP can be explained by a mechanism involving a metal-water-MLHP complex. It is difficult to explain the chemical shift returning to the original value as the reaction progressed if the binding of water with Co^{2+} and hydroperoxides are separate events. The possible role of breakdown products remains to be determined.

This study demonstrated that in a model system the decomposition of MLHP was inhibited by water concentrations of 0.5% to 2.0%, and NMR spectral data provided evidence for water molecules complexing with hydroperoxides and binding with the metal catalysts, Co^{2+} and Cu^{2+}. We propose that formation of a metal-water-hydroperoxide complex is involved in the inhibition by water. Further studies are needed to verify these findings and their interpretation.

TABLE 3

Change in Chemical Shift of NMR Peak of Water *vs* Time in Decomposition of MLHP Catalyzed by Co^{2+}

Time (hr)	Chemical shift (ppm)[a]			
	0.5% H_2O	1.0% H_2O	1.5% H_2O	2.0% H_2O
0.00	2.95	3.00	3.10	3.25
0.25	3.50	3.25	3.30	3.40
1.00	3.35	3.10	3.30	
2.00	3.20			
2.50				3.35
5.00	3.10			
6.00			3.20	3.30
8.00				3.30
9.00	3.00	3.00	3.15	
10.0				3.25
24.0			3.15	

[a]Standard deviations range of the measurements was ±0.007 to 0.014.

ACKNOWLEDGMENTS

We thank the College of Pharmacy and the Department of Chemistry at Washington State University for the use of their proton NMR spectrometer. We also thank Dr. J. Hunt and Dr. H. Dogden, Professors of Chemistry at Washington State University, for their helpful suggestions and criticisms during this study. This study was supported in part by the National Science Foundation, Grant No. CBT-88504518, and the Washington State University Agricultural Research Center, Project 0666.

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ARTICLE

Relationship Between Translocation of Long-Chain Acyl-CoA Hydrolase, Phosphatidate Phosphohydrolase and CTP:Phosphocholine Cytidylyltransferase and the Synthesis of Triglycerides and Phosphatidylcholine in Rat Liver

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Translocation of long-chain acyl-coenzyme A hydrolase from the microsomal fraction to the cytosolic fraction was promoted in cell-free extracts of rat liver by palmitic acid, oleic acid, tetradecylthioacetic acid, and tetradecylthiopropionic acid, and by their CoA esters. The CoA esters were more effective than the non-esterified acids in the translocation of the enzyme. Treatment of normolipidemic rats with sulfur-substituted non-β-oxidizable fatty acid analogues resulted in a transitory increase in hepatic concentration of long-chain acyl-CoA. Longer feeding times almost normalized the hepatic long-chain acyl-CoA content. Microsomal long-chain acyl-CoA hydrolase activity was inhibited, whereas the activity of the cytosolic form was stimulated. The rise in enzyme activity coincided with a reduction in liver content of triglyceride and an increase in hepatic phospholipid content. The results suggest that the activity of long-chain acyl-CoA hydrolase in the cytosol may control the amount of acyl-CoA thioesters in the liver. Esterified and non-esterified fatty acids caused *in vitro* translocation of phosphatidate phosphohydrolase and cytidine 5′-triphosphate (CTP):phosphocholine cytidylyltransferase from the cytosolic fraction to the microsomal fraction. However, the translocation of these two enzyme systems was not obtained *in vivo*. The activity of phosphatidate phosphohydrolase decreased in microsomal and cytosolic fractions while the activity of cytidylyltransferase in these fractions increased. The activities of soluble phosphatidate phosphohydrolase and long-chain acyl-CoA hydrolase appeared to be inversely correlated. The results imply that in cytoplasm, long-chain acyl-CoA hydrolase may compete with the biosynthetic enzymes for the acyl-CoA substrate, thus influencing the rate of lipid synthesis. The reduced hepatic triglyceride content observed in tetradecylthioacetic acid-treated rats is probably due to reduced triglyceride synthesis, which is mediated by an inhibition of phosphatidate phosphohydrolase accompanied with translocation and stimulation of long-chain acyl-CoA hydrolase. Development of fatty liver as an effect of tetradecylthiopropionic acid is probably due to accelerated triglyceride biosynthesis, which is mediated by a stimulation of phosphatidate phosphohydrolase and a decrease in cytosolic palmitoyl-CoA hydrolase activity.
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Abbreviations: BCMTD, 3-thiadicarboxylic acid; CETTD, tetradecylthiopropionic acid; CMOTD, tetradecyloxyacetic acid; CMS, carboxymethyl cellulose; CMTTD, tetradecylthioacetic acid; CoA, coenzyme A; CTP, cytidine 5′-triphosphate; EDTA, (ethylenedinitrolo)tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); P, microsomal fraction; S, soluble fraction.

Long-chain acyl-CoA hydrolase (palmitoyl-CoA hydrolase), phosphatidate phosphohydrolase and cytidine 5′-triphosphate (CTP):phosphocholine cytidylyltransferase are ubiquitous enzymes (1–5). They are found in both the cytoplasm and the cell membrane (6–14). Palmitoyl-CoA hydrolase also occurs in organelles involved in oxidation of fatty acids such as mitochondria (15,16) and peroxisomes (16–18), and its distribution between the different cell compartments can be modified (19). This has been observed in the rat liver after starvation or repeated administration of peroxisome proliferators such as clofibrate, tiadenol (17,20), and partially hydrogenated fish oils (21,22), conditions which increase the hepatic concentration of long-chain acyl-CoA (19,23). Thus, increased long-chain acyl-CoA hydrolase activity in cytosol during peroxisome proliferation is apparently a consistent phenomenon, exhibiting a close correlation with induction of peroxisomal β-oxidation (14).

Treatment of normolipidemic rats with sulfur-substituted non-β-oxidizable fatty acids, which are peroxisome proliferators (19), results in a reduction in the liver content of triglycerides (24) and an increase in phospholipid levels (25). This is accompanied by an increase in CTP:phosphocholine cytidylyltransferase activity and a decrease in phosphatidate phosphohydrolase activity. The results may indicate that the biosynthesis of triglycerides and phospholipids are coordinately regulated (25).

Both phosphatidate phosphohydrolase and CTP:phosphocholine cytidylyltransferase have been reported to exist in two interconvertible forms—a microsomal form and a cytosolic form (1,2,8–11). It is thought that the cytosolic form, which is a functionally inactive reservoir, can become metabolically activated when translocated to the endoplasmic reticulum in which the synthesis of glycerolipids occurs (7,8). As long-chain fatty acids and their CoA esters are able to promote translocation of these two enzymes, increased synthesis of glycerolipids, particularly triglycerides, may help to limit the rise in the concentration of fatty acyl-CoA esters in the liver (2,26) and prevent them from becoming toxic. If the rise in concentration of fatty acyl-CoA esters is not limited, the formation of a fatty liver can result.

The most dramatic effect observed after the administration of 3-tetradecylpropionic acid, which is able to undergo one cycle of β-oxidation, was the development of fatty liver (19). Since long-chain acyl-CoA hydrolase also appears to be important in the regulation of long-chain acyl-CoA, the aim of the present investigation was to examine whether *in vitro* translocation also occurs for this enzyme. We were also interested in determining whether the 3- and 4-sulfur substituted fatty acids and their CoA esters interfere with the translocation process of phosphatidate phosphohydro-

lase and CTP:phosphocholine cytidylyltransferase. In addition, we examined whether changes in the activities of phosphatidate phosphohydrolase, CTP:phosphocholine cytidylyltransferase and long-chain acyl-CoA hydrolase activities and their subcellular distribution are affected by and related to the increase in concentration of hepatic long-chain acyl-CoA by feeding 3-thiadicarboxylic acid, tetradecylthioacetic acid and tetradecylthiopropionic acid to rats.

The present data support the concept that induction of long-chain acyl-CoA hydrolase activity, particularly in the cytosolic form, is a protective adaption against unfavorable levels of acyl-CoA. Furthermore, our results imply that acyl-CoA hydrolase may compete with the biosynthetic enzymes for the acyl-CoA substrate, thus influencing the rate of lipid synthesis.

MATERIALS AND METHODS

Chemicals and drugs. [1-^{14}C]Palmitoyl-CoA and [^{3}H]-glycerol 3-phosphate were purchased from the Radiochemical Centre (Amersham, England). 1,10-*Bis*(carboxymethylthio)decane (3-thia-dicarboxylic acid) (BCMTD), tetradecylthioacetic acid (CMTTD), tetradecylthiopropionic acid (CETTD) and the CoA esters of the monocarboxylic acids were prepared as described earlier (27). The synthesis of tetradecyloxyacetic acid also has been described previously (J. Skorve, D. Asiedu, M. Solbakken, R.K. Berge, unpublished data). Table 1 shows the structure of the different compounds. All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and treatments. Male Wistar rats from Møllegaard Breeding Laboratory (Ejby, Denmark), weighing 170–180 g, were housed individually in metal wire cages in a room maintained at 12-hr light-dark cycles at a constant temperature of 20 ± 3°C. The animals were acclimatized for at least one week under these conditions before the start of the experiment. The fatty acid analogues were suspended in 0.5% sodium carboxymethyl cellulose (CMS) and administered by gastric intubation in a volume of 1 mL once a day at a dose of 150 mg/day/kg body weight. The control animals received only CMS. All animals had free access to water and food (17,19). In some experiments the rats were fed *ad libitum* the diet supplemented with tetradecyloxyacetic acid at a dose of 400 mg/day/kg body weight for 5 days.

Preparation and treatment of subcellular fractions. The livers from individual rats were homogenized in ice-cold sucrose-medium (0.25 M sucrose in 10 mM HEPES buffer, pH 7.4 and 1 mM EDTA), and the resulting nuclear and postnuclear fraction were used as the total homogenate (19). For further analytical differential centrifugation experiments, postnuclear fractions from three animals were pooled (19,24,25) before microsomal and cytosolic enriched fractions were isolated. Variation in the response from animal to animal was estimated separately in the group of control animals for selected enzymes and lipids.

A postperoxisomal fraction obtained from rat liver homogenates after centrifugation using a time integral of 4.3×10^9 min^{-1} (t = 30 min, r_{min} = 4.8 cm and r_{max} = 14.6 cm) (16,21) was incubated with various fatty acids and CoA esters with or without bovine serum albumin for 10 min at 37°C (7,8). Microsomal and cytosolic fractions were isolated after centrifugation at a time integral of 7.4×10^{10} min^{-1} (t = 60 min, r_{min} = 6.6 cm and r_{max} = 15.2) at 4°C (7,8,16,21).

Analytical methods. Protein was assayed with Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). The enzymatic activities of CTP:phosphocholine cytidylyltransferase (28), and long-chain acyl-CoA hydrolase (using palmitoyl-CoA as substrate) at a concentration of 60 μM (5,19) were determined as earlier described. The phosphatidate phosphohydrolase activity was measured as phosphate release in the absence and presence of 5 mM $MgCl_2$ (29). The activity of the phosphohydrolase was also measured as diacylglycerol formation. The assay conditions were identical in the presence of 5 mM $MgCl_2$, as earlier described (29). The reaction was stopped by adding 1 mL of chloroform/methanol (1:2, v/v), and the diacylglycerol formed was extracted into the chloroform phase. The diacylglycerol was quantitated with a diacylglycerol assay reagent system from Amersham. Free CoASH and acid-insoluble CoA (the CoA-thioesters of native fatty acids and/or the CoA thioesters of the drugs) were measured in liver extracts processed as described previously (23).

RESULTS

Translocation of long-chain acyl-CoA hydrolase from membrane to cytoplasm in vitro. The addition of increasing concentrations of oleoyl-CoA to a post-light mitochondrial fraction of rat liver caused a progressive decrease in specific activity of palmitoyl-CoA hydrolase in the microsomal fraction (Fig. 1). By comparison, oleoyl-CoA increased the activity in the soluble fraction (Fig. 1). Palmitoyl-CoA was comparable to oleoyl-CoA in this respect (Table 2), but the CoA esters of the sulfur-substituted fatty acid analogues, tetradecylthioacetic acid and tetradecylthiopropionic acid, were more effective than the CoA esters of "normal" fatty acids in facilitating the translocation (Table 2). Clofibroyl-CoA was less effective in facilitating the translocation when compared with controls to which

TABLE 1

Structure and Abbreviations of the Sulfur- and Oxysubstituted Fatty Acid Analogues

CH_3-$(CH_2)_{13}$-S-CH_2COOH	Tetradecylthioacetic acid	CMTTD
CH_3-$(CH_2)_{13}$-S-CH_2-CH_2COOH	Tetradecylthiopropionic acid	CETTD
HOOC-CH_2-S-$(CH_2)_{10}$-S-CH_2COOH	1,10-*Bis*(carboxymethylthio)decane (3-thiadicarboxylic acid)	BCMTD
CH_3-$(CH_2)_{13}$-O-CH_2COOH	Tetradecyloxyacetic acid	CMOTD

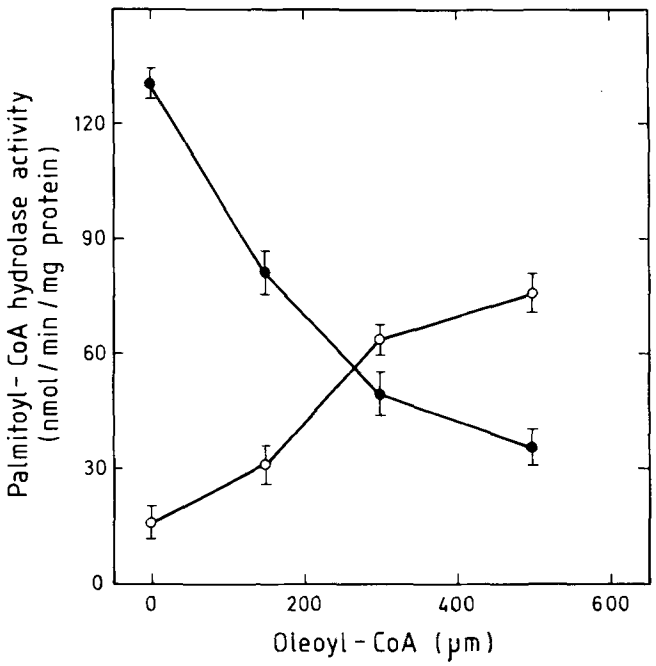

FIG. 1. Effect of oleoyl-CoA on the activity of long-chain acyl-CoA hydrolase in the microsomal (●) and soluble (○) fraction of rat liver. The post-light mitochondrial supernatant of rat liver was incubated for 10 min at 37°C at the concentrations of oleoyl-CoA indicated. The values are the means ± SD of three independent experiments. For details, see Materials and Methods.

no fatty acid had been added. Evidence is presented in Table 2 that the unesterified fatty acids were less effective than their CoA esters in promoting the translocation of palmitoyl-CoA hydrolase from a membrane-associated compartment to the soluble fraction. In the presence of albumin, palmitoyl-CoA and oleoyl-CoA caused increased specific activity of palmitoyl-CoA hydrolase in the cytosolic fraction (Table 2). Furthermore, addition of fatty acyl-CoA at concentrations similar to those generated *in vivo* by feeding the various fatty acids (Table 4) was effective in facilitating the translocation of palmitoyl-CoA hydrolase (Table 3). Thus, the rlease of long-chain acyl-CoA hydrolase from microsomes is not simply an artefact due to the detergent action of the CoA-esters.

The activity of rotenone-insensitive NADPH-cytochrome c reductase in the cytosolic fraction was less than 2% of the activity present in the liver homogenates. The activity of lactate dehydrogenase in the particulate fraction after resuspension and centrifugation was less than 2% of the total activity present in the homogenate. No increased reductase activity in the cytosolic fraction was observed after addition of the different CoA esters. Thus, the low cross contaminations were irrelevant for the measurement of the cellular distribution of palmitoyl-CoA hydrolase. The CoA esters seem to release certain microsome-associated proteins more or less selectively.

Translocation of long-chain acyl-CoA hydrolase from membrane to cytoplasm in vivo. Treatment of rats with 3-thiadicarboxylic acid and tetradecylthioacetic acid at a dose of 150 mg/day/kg body weight for 5 days resulted

TABLE 2

Effects of Fatty Acid and their CoA Esters on the Activity of Long-Chain Acyl-CoA Hydrolase in the Microsomal and Cytosolic Fractions in Liver of Untreated Rats[a]

	Long-chain acyl-CoA hydrolase activity					
	Microsomal fraction (P)			Soluble fraction (S)		
Additions	nmol/min/mg protein	nmol/min	%	nmol/min/mg protein	nmol/min	%
None (control)	116.7 ± 5.4	590.2 ± 46.2	100	16.2 ± 0.3	289.2 ± 10.5	100
+ Palmitoyl-CoA (300 μM)	39.8 ± 10.8[b]	169.0 ± 52.1[b]	28.6 ± 8.8[b]	42.7 ± 3.2[b]	757.2 ± 55.3[b]	261.8 ± 19.1[b]
+ Palmitoyl-CoA (300 μM) + albumin (0.2 mg/mL)	53.0 ± 4.2[b]	265.2 ± 30.6[b]	44.9 ± 5.2[b]	36.3 ± 4.8[b]	598.5 ± 35.2[b]	207.0 ± 12.2[b]
+ Oleoyl-CoA (300 μM)	27.7 ± 1.4[b]	133.2 ± 25.0[b]	22.6 ± 4.2[b]	33.7 ± 4.6[b]	606.5 ± 50.2[b]	209.7 ± 17.3[b]
+ Oleoyl-CoA (300 μM) + albumin (0.2 mg/mL)	52.6 ± 4.5[b]	225.6 ± 41.0[b]	38.2 ± 6.9[b]	30.1 ± 5.3[b]	596.6 ± 43.0[b]	206.3 ± 14.8[b]
+ Palmitic acid (300 μM)	94.3 ± 10.6[b]	397.0 ± 36.1[b]	67.3 ± 6.1[b]	20.8 ± 2.6[b]	420.1 ± 36.8[b]	145.3 ± 12.7[b]
+ Tetradecylthioacetic acid (300 μM)	106.8 ± 5.2	571.6 ± 51.2	96.8 ± 8.7	17.4 ± 0.8	291.8 ± 40.1	100.8 ± 13.9
+ Tetradecylthiopropionic acid (300 μM)	101.1 ± 3.8	563.5 ± 10.8	95.5 ± 2.8	17.3 ± 1.4	289.1 ± 15.6	100.0 ± 5.4
+ Tetradecylthioacetic acid-CoA (300 μM)	11.3 ± 0.3[b]	44.8 ± 0.4[b]	7.6 ± 0.7[b]	41.5 ± 0.6[b]	751.3 ± 30.0[b]	359.8 ± 10.3[b]
+ Tetradecylthiopropionic acid-CoA (300 μM)	15.2 ± 0.7[b]	63.8 ± 3.1[b]	10.8 ± 0.5[b]	46.3 ± 1.2[b]	847.0 ± 9.0[b]	292.9 ± 3.1[b]
+ Clofibroyl-CoA (300 μM)	116.8 ± 4.1	590.2 ± 25.7	100.0 ± 4.4	18.3 ± 0.7	312.7 ± 6.4	108.1 ± 2.2

[a] Post-light mitochondrial fraction was incubated with the fatty acids and their CoA esters with or without albumin, as indicated, for 10 min at 37°C. Microsomal and soluble fractions were then prepared as described in Materials and Methods. The hydrolase activity was measured with palmitoyl-CoA as substrate at a concentration of 60 μM. The total hydrolase activity in microsomal and soluble fractions relative to the post-mitochondrial fraction was in the range of 96 to 111%. The tabulated values are expressed as means ± ranges of two independent experiments.

[b] $P < 0.05$ between control (no addition) and addition.

TABLE 3

Effects of CoA Esters on the Activity of Long-Chain Acyl-CoA Hydrolase in the Microsomal and Cytosolic Fractions of Liver in Alkyloxyacetic Acid (400 mg/day/kg body weight) Treated Rats for Five Days[a]

Additions	Long-Chain acyl-CoA hydrolase activity					
	Microsomal fraction (P)			Soluble fraction (S)		
	nmol/min/mg protein	nmol/min	%	nmol/min/mg protein	nmol/min	%
None (control)	86.3 ± 1.5	254.5 ± 33.5	100	28.1 ± 1.1	421.1 ± 5.6	100
+ Palmitoyl-CoA (300 mM)	36.5 ± 4.2[b]	72.9 ± 5.6[b]	28.6 ± 2.2[b]	36.0 ± 2.5[b]	554.5 ± 30.5[b]	131.7 ± 7.2[b]
+ Tetradecylthioacetic acid-CoA						
(75 μM)	71.2 ± 1.1[b]	209.3 ± 20.6	82.2 ± 8.1	30.1 ± 0.6	460.0 ± 40.2	109.3 ± 9.5
(150 μm)	43.2 ± 3.1[b]	96.0 ± 5.1[b]	37.7 ± 2.0[b]	36.0 ± 2.1[b]	527.3 ± 80.1	125.2 ± 19.0
+ Tetradecyltiopropionic acid-CoA						
(75 μM)	66.9 ± 2.1[b]	173.5 ± 10.2[b]	68.2 ± 4.0	30.2 ± 0.5	450.3 ± 10.6	106.9 ± 2.6
(12 μM)	78.6 ± 4.2	256.1 ± 8.6	100.6 ± 3.4	28.7 ± 0.4	390.4 ± 14.1	92.7 ± 3.3

[a]Post-light mitochondrial fraction was incubated with various additions for 10 min at 37°C. Microsomal and soluble fractions were then prepared as described in Materials and Methods. The hydrolase activity was measured with palmitoyl-CoA as substrate at a concentration of 60 μM. The total hydrolase activity in microsomal and soluble fractions relative to control (no additions) was in the range of 92 to 99%. The hydrolase activities are calculated relative to those with no addition equaling 100%.

[b]$P < 0.05$ between control and additions.

TABLE 4

Effect of Sulfur-Substituted Fatty Acid Analogues on the Level of CoASH Derivatives and Long-Chain Acyl-CoA Hydrolase Activity in Liver of Rats[a]

Treatment	Days of feeding	Long-chain acyl-CoA hydrolase		Long-chain acyl-CoA		Ratio long-chain acyl-CoA
		Microsomes	Cytosol	nmol/g liver	CoASH	CoASH
Control		100	100	29.6 ± 6.2	65.6 ± 4.6	0.45
Tetradecylthioacetic acid	1/2	90.6 ± 5.2	120.9 ± 8.6[b]	91.4 ± 4.6[b]	54.5 ± 4.5	1.68
	2	95.5 ± 4.8	146.1 ± 4.6[b]	147.1 ± 10.9[b]	70.2 ± 8.9	2.10
	3	92.6 ± 3.1	149.1 ± 4.6[b]	105.8 ± 5.3[b]	78.4 ± 3.0	1.35
	7	89.5 ± 2.7[b]	159.3 ± 3.6[b]	56.2 ± 0.6[b]	105.2 ± 15.4[b]	0.54
3-Thiadicarboxylic acid	1	85.3 ± 4.1[b]	168.3 ± 4.8[b]	92.7 ± 20.5[b]	69.8 ± 3.6	1.33
	2	93.2 ± 3.1[b]	233.5 ± 3.6[b]	144.9 ± 30.1[b]	91.6 ± 8.6[b]	1.58
	3	85.5 ± 1.9[b]	356.9 ± 2.8[b]	39.7 ± 8.5	125.9 ± 10.2[b]	0.32
	7	83.7 ± 4.2[b]	641.9 ± 8.6[b]	51.1 ± 10.1[b]	201.1 ± 15.6[b]	0.25
Tetradecylthiopropionic acid	1	80.2 ± 5.2[b]	78.4 ± 4.1[b]	30.1 ± 2.2	61.8 ± 3.1	0.49
	3	78.3 ± 3.6[b]	76.6 ± 3.2[b]	31.6 ± 6.2	78.4 ± 14.1	0.40
	7	75.3 ± 4.5[b]	66.5 ± 6.1[b]	38.6 ± 9.1	77.6 ± 13.6	0.50
	14	69.9 ± 2.6[b]	96.9 ± 4.2	61.6 ± .8[b]	61.4 ± 5.2	1.00

[a]The tabulated values of CoA derivatives (nmol/g liver) represent means ± SD of 12 control animals and 3 rats of 2 feeding groups. The acyl-CoA hydrolase activities are presented relative to those of control animals equaling 100% (n = 6). In controls the mean acyl-CoA hydrolase activity of the microsomal and soluble fractions was 154.4 ± 2.8 nmol/min/mg protein and 16.8 ± 2.8 nmol/min/mg protein, respectively.

[b]$P < 0.02$ between control and treated rats.

in a progressive increase in the activity of cytosolic palmitoyl-CoA hydrolase accompanied by decreasing microsomal enzyme activity (Table 4). Administration of palmitic acid was less effective in facilitating the translocation, but at higher palmitic acid doses increased activity of palmitoyl-CoA hydrolase in the cytosolic fraction was observed (30,31). The activity of the enzyme in the microsomal fraction was decreased (31). Administration of tetradecylthiopropionic acid decreased the activities of microsomal and cytosolic long-chain acyl-CoA hydrolase (Table 4).

The results of Table 4 show that repeated administration of 3-thiadicarboxylic acid and tetradecylthioacetic acid increased the hepatic concentration of long-chain acyl-CoA, which was already apparent during the first days of treatment. Increased CoASH levels were observed within longer feeding periods and the ratio of long-chain acyl-CoA to CoASH was maximally increased after two days of feeding. Subsequently, after seven days of feeding 3-thiadicarboxylic acid, the ratio was essentially normalized (Table 4). In agreement with earlier observations (30), no redistribution of long-chain acyl-CoA and free CoASH

was observed in tetradecylthiopropionic acid treated animals (Table 4). Repeated administration of the β-oxidizable sulfur acid tended to increase the concentration of long-chain acyl-CoA after 14 days of feeding (Table 4).

Translocation of phosphatidate phosphohydrolase and CTP:phosphocholine cytidylyltransferase to the microsomal fraction in vitro. The activities of Mg^{2+}-dependent phosphatidate phosphohydrolase (Table 5), as well as CTP:phosphocholine cytidylyltransferase (Table 6), were increased in the microsomal fraction after addition of esterified or non-esterified fatty acids. In most cases, the CoA esters of the thio fatty acids, as well as the CoA esters of "normal" fatty acids, were more effective than the non-esterified fatty acids in stimulating the enzyme activities (Tables 5 and 6). In contrast, the activities of the cytosolic forms of phosphatidate phosphohydrolase (Table 5) and CTP:phosphocholine (Table 6) tended to decrease after additions of CoA esters. Thus, a translocation of phosphatidate phosphohydrolase and CTP:phosphocholine cytidylyltransferase from the cytosolic to the microsomal fraction

TABLE 5

Effect of Fatty Acids and their CoA Esters on the Activity of Phosphatidate Phosphohydrolase in the Microsomal and Cytosolic Fractions of Rat Liver[a]

	Phosphatidate phosphohydrolase (nmol/min/mg protein)					
	Phosphate formation				Diacylglycerol formation	
	Soluble fraction		Microsomal fraction			
	− $MgCl_2$	+ $MgCl_2$	− $MgCl_2$	+ $MgCl_2$	Soluble fraction	Microsomal fraction
None (control)	2.6 ± 0.2	6.7 ± 0.3	9.1 ± 0.1	10.3 ± 0.4	10.5 ± 0.4	8.3 ± 0.6
+ Palmitic acid	2.5 ± 0.1	6.1 ± 0.4	13.8 ± 0.3[b]	11.1 ± 2.6	ND[c]	ND
+ Palmitoyl-CoA	2.2 ± 0.1	6.1 ± 0.2	15.2 ± 0.2[b]	11.9 ± 0.8[b]	9.1 ± 0.4	16.4 ± 1.2[b]
+ Oleoyl-CoA	2.3 ± 0.1	7.3 ± 0.4	13.9 ± 0.3[b]	10.8 ± 0.5	9.9 ± 0.4	12.7 ± 0.5[b]
+ Tetradecylthioacetic acid	2.6 ± 0.2	7.1 ± 0.6	14.4 ± 0.5[b]	11.5 ± 1.6	11.0 ± 0.6	11.9 ± 1.2[b]
+ Tetradecylthioacetic acid-CoA	2.1 ± 0.1[b]	5.8 ± 0.4	15.7 ± 0.1[b]	11.4 ± 0.2[b]	10.1 ± 1.4	15.9 ± 1.2[b]
+ Tetradecylthiopropionic acid	2.4 ± 0.2	7.5 ± 0.5	14.1 ± 0.4[b]	10.8 ± 0.3	10.4 ± 0.5	10.5 ± 0.3[b]
+ Tetradecylthiopropionic acid-CoA	2.2 ± 0.2	5.5 ± 0.5	15.6 ± 0.2[b]	11.1 ± 0.2	9.1 ± 0.5	14.8 ± 1.6[b]
+ Clofibroyl-CoA	2.3 ± 0.1	7.3 ± 1.2	13.8 ± 0.3[b]	11.7 ± 0.8	10.3 ± 0.8	13.3 ± 0.4[b]

[a]The post-light mitochondrial fraction was incubated with various additions at a concentration of 250 μM. Microsomal and cytosolic fractions were then prepared as described in Materials and Methods. Enzyme activities (nmol/min/mg protein) are expressed as mean ± ranges of two independent experiments.

[b]$P < 0.05$ between control and additions.

[c]ND, not determined.

TABLE 6

Effect of Fatty Acids and their CoA Esters on the Activity of CTP:Phosphocholine Cytidylyltransferase in the Microsomal and Cytosolic Fractions of Rat Liver[a]

Additions	CTP:phosphocholine cytidylyltransferase (nmol/min/mg protein)		Total enzyme activity in S+P relative to control (no additions) %
	Soluble fraction	Microsomal fraction	
None (control)	0.46 ± 0.03	0.20 ± 0.02	100
+ Palmitic acid	0.38 ± 0.04	0.26 ± 0.01[b]	94 ± 2
+ Palmitoyl-CoA	0.41 ± 0.02	0.32 ± 0.03[b]	102 ± 5
+ Oleoyl-CoA	0.40 ± 0.03	0.24 ± 0.04	101 ± 2
+ Tetradecylthioacetic acid	0.44 ± 0.05	0.24 ± 0.02	94 ± 3
+ Tetradecylthioacetic acid-CoA	0.35 ± 0.02[b]	0.33 ± 0.02[b]	97 ± 6
+ Tetradecylthiopropionic acid	0.45 ± 0.04	0.25 ± 0.05	104 ± 3
+ Tetradecylthiopropionic acid-CoA	0.38 ± 0.04[b]	0.37 ± 0.02[b]	101 ± 4
+ Clofibroyl-CoA	0.42 ± 0.01	0.25 ± 0.02	92 ± 4

[a]The post-light mitochondrial fraction was incubated with various additions at a concentration of 250 μM. Microsomal and cytosolic fractions were then prepared as described in Materials and Methods. Enzyme activity are expressed as mean ± ranges of two independent experiments.

[b]$P < 0.05$ between control and additions.

TABLE 7

Time-Dependent Changes of Phosphatidate Phosphohydrolase and CTP:Phosphocholine Cytidylyltransferase Activities in Cytosolic (S) and Microsomal (P) Fractions of Rats Treated with 3-Thiadicarboxylic Acid and Tetradecylthioacetic Acid at a Dose of 150 mg/day/kg Body Weight[a]

Treatment	Enzymes	Fractions	Time of exposure (days)				
			0	1	3	7	10
3-Thiadicarboxylic acid	Phosphatidate Phosphohydrolase	P	100	80 ± 5[b]	79 ± 6[b]	70 ± 6[b]	70 ± 6[b]
		S	100	78 ± 6[b]	56 ± 8[b]	45 ± 6[b]	48 ± 6[b]
Tetradecylthioacetic acid		P	100	95 ± 4	79 ± 5[b]	70 ± 8[b]	75 ± 5[b]
		S	100	92 ± 6	88 ± 4[b]	77 ± 6[b]	63 ± 8[b]
3-Thiadicarboxylic acid	CTP:Phosphocholine transferase	P	100	105 ± 8	169 ± 10[b]	176 ± 8[b]	202 ± 10[b]
		S	100	101 ± 6	132 ± 8[b]	142 ± 6[b]	176 ± 7[b]
Tetradecylthioacetic acid		P	100	129 ± 8	135 ± 6[b]	186 ± 11[b]	152 ± 12[b]
		S	100	107 ± 6	120 ± 5[b]	121 ± 10[b]	132 ± 6[b]

[a]The enzyme activities are presented relative to those of control animals equaling 100%. The tabulated values are the means ± SD of 12 control animals and 6 treated rats. In control animals the mean phosphatidate phosphohydrolase activity in the microsomal and cytosolic fractions was 13.9 ± 1.8 nmol/min/mg protein and 7.1 ± 1.4 nmol/min/mg protein, respectively. The mean CTP:phosphocholine cytidylyltransferase activity in the microsomal and cytosolic fractions was 4.2 ±0.2 nmol/min/g liver and 44.1 ± 3.0 nmol/min/g liver, respectively.

was promoted in the cell-free extract of rat liver by fatty acid and their CoA esters (Tables 5 and 6). In addition, evidence is presented in Tables 5 and 6 that both enzymes can translocate from cytosol to microsomes *in vitro* in the presence of added esterified or unesterified sulfur-substituted fatty acid analogues. Clofibroyl-CoA was less effective than tetradecylthioacetic-CoA and tetradecylthiopropionic-CoA in facilitating the translocation (Tables 5 and 6).

Cellular distribution of phosphatide phosphohydrolase and CTP:phosphocholine cytidylyltransferase in vivo. Repeated administration of 3-tetradecylthioacetic acid and 3-thiadicarboxylic acid caused a decrease in the activities of microsomal and cytosolic Mg^{2+}-dependent phosphatidate phosphohydrolase (Table 7), whereas the microsomal and soluble CTP:phosphocholine cytidylyltransferase activities were stimulated. Thus, no translocation *in vivo* of these enzymes from the cytosolic compartment to the microsomal compartment was observed after repeated administration of sulfur-substituted fatty acid analogues.

DISCUSSION

In agreement with earlier observations (3–11), esterified and unesterified fatty acids can cause phosphatidate phosphohydrolase (Table 5) and CTP:phosphocholine cytidylyltransferase (Table 6) to translocate from the cytosol and to associate with microsomal membranes *in vitro*. The present results demonstrate that the two enzymes also can be translocated to the endoplasmic reticulum by sulfur-substituted fatty acid analogues. The CoA-esters of these peroxisome proliferating acids appeared to be more effective than their unesterified acids in facilitating the translocation (Table 5).

Translocation of phosphatidate phosphohydrolase and CTP:phosphocholine cytidylyltransferase *in vivo* could not be demonstrated in our study (Table 7). Moreover, the phosphohydrolase activity was inhibited in 3-thiadicarboxylic acid and tetradecylthioacetic acid treated animals despite the increase in long-chain acyl-CoA content (Table 4). The reason for the discrepancy *in vivo* compared to the *in vitro* studies is not clear. It cannot be explained by the effect of Mg^{2+} or the assay conditions, as decreased cytosolic and microsomal phosphatidate phosphohydrolase activity *in vivo* was observed in the absence of Mg^{2+} (assayed as phosphate formation) (29) and in the presence of Mg^{2+} (assayed as diacylglycerol formation) (Table 7). Furthermore, in the presence of Mg^{2+}, measured phosphate formation equaled diacylglycerol formation in most cases (Table 5). However, the activity of Mg^{2+}-independent phosphatidate phosphohydrolase was much lower than the Mg^{2+}-dependent enzyme activity in the cytosolic fractions (Table 5). As subcellular fractionation itself causes no redistribution of the phosphohydrolase, observed differences between *in vivo* and *in vitro* data suggest that other mechanisms for stimulation and inhibition must also be considered.

The present study confirms earlier observations that treatment with sulfur-substituted fatty acid analogues, which are classified as hypolipidemic peroxisome proliferating agents (19,24), cause inhibition of the phosphatidate phosphohydrolase activity coupled with stimulated CTP:phosphocholine cytidylyltransferase activity (25). In our study, an inhibition of the latter enzyme activity was accompanied by a stimulation of the former (Table 7). Thus, a coordinated regulation of triglyceride and phospholipid biosynthesis appears to exist (25). Taken together, the activity of phosphatidate phosphohydrolase may be important in regulating the rate of triglyceride synthesis (25). However, it is not sufficient to limit the rise in the concentration of acyl-CoA esters in the liver (Table 4), as has been suggested (7,26).

The present study demonstrates that esterified and unesterified fatty acids and sulfur-substituted fatty acids may promote translocation of long-chain acyl-CoA hydrolase from the microsomal fraction to the cytosolic fraction (Fig. 1 and Table 2). The CoA esters were more effective than the free acids (Table 2). The *in vitro* effects observed with fatty acyl-CoA derivatives, particularly as

they affect the release, could simply be artefacts that occur at the concentrations used, *e.g.*, due to detergent action, certain microsome-associated proteins may be released more or less selectively. However, relatively low concentrations of CoA esters (Table 3), at *in vitro* fatty acyl-CoA concentrations similar to those generated upon *in vivo* feeding of the various acids (Table 4) (30,31), were able to promote translocation of long-chain acyl-CoA hydrolase (Table 3).

Changes in enzyme activities can be attributed to inhibition or activation as well as to compartmental translocation. Fatty acyl-CoA hydrolase activity can be changed by treating the *in vitro* systems with high concentrations of the same and different CoA esters. The hydrolase assay was run at an optimal palmitoyl-CoA concentration, and addition of different esterified and non-esterified CoA esters did not interfere with the assay; recoveries of enzyme activities in soluble and microsomal fractions were close to 100% (between 92 and 111%). The changes in long-chain acyl-CoA hydrolase activity do not appear to involve inhibition or activation of the enzyme, but more likely translocation between compartments.

The *in vivo* data on redistribution of long-chain acyl-CoA hydrolase between microsomal and cytosolic fractions, which may be promoted by long-chain acyl-CoA (Table 4), were comparable with the *in vitro* data (Tables 2 and 3). Treatment with tetradecylthiopropionic acid resulted in no changes in the distribution of CoASH between the free and acylated forms, and in no redistribution of the hydrolase activities (30) (Table 4). Both the microsomal and cytosolic hydrolase activities were decreased (Table 4). This suggests that the activity of long-chain acyl-CoA hydrolase in the cytosol may control the amount of acyl-CoA ester in the liver.

Reduction in the triglyceride content of the liver and an increase in hepatic phospholipid concentrations have been observed in 3-thiadicarboxylic acid and tetradecylthioacetic acid treated rats (25). In contrast, repeated administration of tetradecylthiopropionic acid led to a drastic increase in the hepatic triglyceride content. It is conceivable that increased activity of cytosolic long-chain acyl-CoA hydrolase may limit the increase in the concentration of long-chain acyl-CoA and, thereby may protect the cells against a toxic detergent effect and formation of fatty liver.

The rise and fall in long-chain acyl-CoA hydrolase activity in cytosol (Table 4) coincided with a decreased and increased concentration of hepatic triglycerides, respectively (25). Thus, the long-chain acyl-CoA hydrolase in cytoplasm may compete with the biosynthetic enzymes for the acyl-CoA substrate, thus influencing the rate of lipid synthesis. The reduced hepatic triglyceride content observed in non-β-oxidizable fatty acid-treated rats is probably due to reduced triglyceride synthesis which is mediated by translocation and stimulation of long-chain acyl-CoA hydrolase and inhibition of phosphatidate phosphohydrolase. Development of fatty liver of tetradecylthiopropionic acid (24,25) is probably due to accelerated triglyceride synthesis which is mediated by an increase in the availability of CoA esters along with enzymatic inhibition (long-chain acyl-CoA hydrolase) (Table 4) and mitochondrial β-oxidation (25,30,31) and stimulation (phosphatidate phosphohydrolase) (25). Thus, the palmitoyl-CoA hydrolase activity may play an important role in regulating substrate availability, *i.e.*, fatty acids, as a major determinant of the rate of triglyceride biosynthesis. To what extent the palmitoyl-CoA hydrolase serve as an intracellular buffer preventing great fluctuation in the concentration of fatty acyl-CoA is presently unknown.

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Transfer of Liposomes Containing Dolichol into Isolated Hepatocytes

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Isolated rat hepatocytes were preincubated with egg lecithin liposomes containing [^{3}H]dolichol and [^{3}H]-dolichyl ester, and the intracellular levels and distributions of these lipids were subsequently determined after incubation in a liposome-free medium. [^{3}H]Dolichol was recovered initially mainly in microsomes, and no increase with time in the low level of this compound in the mitochondrial/lysosomal fraction could be observed. A small portion of the labeled dolichol was esterified in the endoplasmic reticulum and transferred to the lysosome-containing fraction. [^{3}H]Dolichyl linoleate was initially localized in microsomes and supernatant, but later accumulated in the mitochondria/lysosomes. Dolichyl linoleate was found in the membrane of microsomes, in the membrane and lumen of lysosomes, and in the soluble cytoplasm. Exogenous dolichol recovered in microsomes was not phosphorylated to any significant extent. Liposomal phosphatidylcholine also showed preferential accumulation in microsomes after incubation with hepatocytes. These results indicate that exogenous or endogenously formed dolichyl esters are transferred from the endoplasmic reticulum to lysosomes, probably through the cytoplasm. It appears that fatty acids play a role in targeting these lipids to their intracellular locations.
Lipids 27, 248-254 (1992).

Dolichol is synthesized in the endoplasmic reticulum and also in the peroxisomes, but it is present in all membranes and in the cytoplasm (1-4). Studies to date have concentrated on the details of the biosynthetic mechanism and the influence of dolichol on stability, fluidity and permeability in model membranes and on its stimulating effects on membrane fusion (5-8). The phosphorylated form is an obligatory intermediate in glycoprotein synthesis, which has been studied in great detail (9).

The broad distribution of dolichol, its rapid rate of synthesis and the large variation in the amounts present under different experimental conditions indicate that the uptake and intracellular transport of dolichols are important factors which appear to contribute to the homeostasis of cell life. It seems of importance to study these processes using a suitable experimental system in which uptake can be followed. Isolated hepatocytes take up lipids from liposomes in the medium and a large portion of this exogenous lipid becomes subsequently associated with intracellular organelles (10). The components of liposomes may be internalized by a process involving the uptake of individual components, instead of entire particles. The uptake of micellar lipids depends greatly on the cell type, and lipid uptake by many cells in culture proceeds rather slowly (11).

Using such a system, several questions concerning the intracellular fate of dolichol can be answered. These include how dolichol initially distributes in the cell, how it is redistributed from its initial location to other membranes, whether it is incorporated into membranes or distributed within the organelle and, finally, whether dolichol is modified during uptake or intracellular transport.

The receptor-mediated uptake of low density lipoproteins by hepatocytes has been studied extensively (12). In this process, lipids enter the cell *via* the phagosomal-lysosomal system, where they are processed before discharge into the cytoplasm and before further translocation into intracellular membrane systems. When dolichol is injected into the blood of rats, it ends up in the phagosomal hepatic lysosomes, where it remains for at least 6 days without being transferred to other organelles (13).

Dolichol transport occurs not only within the cell, but dolichol is also discharged through the endoplasmic reticulum (ER)-Golgi system into the blood, where it participates in the formation of high density lipoprotein (14). Like cholesterol, dolichol appears in the bile, and this route of excretion is believed to make a major contribution to dolichol turnover (15,16). The half-life of dolichol in the endoplasmic reticulum is about 80-120 hr, while in lysosomes it ranges between 120 and 140 hr (17). These half-lives are about the same as those found for other neutral and phospholipids, suggesting that active breakdown of polyisoprenoids may occur in the liver. In fact, breakdown products of dolichol were recently found in bile (18,19). Attempts to identify metabolites of this process have so far not been successful (20,21). Uptake of extracellular dolichol and its subsequent intracellular translocation may provide a system for investigating the breakdown process (10,11).

In a number of physiological and pathophysiological conditions, such as in aging (22-25), under drug treatments (26), exposure to plasticizer (27), and under various experimental (28-30) and human pathological conditions (31-34), considerable alterations in the amount, nature and distribution of polyisoprenoids in cells and tissues are observed. Obviously, the uptake, transport and discharge of dolichols can be considerably modified by an as-yet-unknown mechanism.

In the present study, we have incubated isolated rat hepatocytes with egg lecithin liposomes containing polyisoprenoids. The uptake and distribution of the lipids in the membranes and the lumen of subcellular organelles were analyzed and metabolic modifications of dolichol were investigated.

MATERIALS AND METHODS

Animals and cells. In all experiments, livers of non-starved Sprague-Dawley rats (330-360 g) were used. For the isolation of hepatocytes, the livers were first perfused with

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Abbreviations: EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ER, endoplasmic reticulum; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

150 mL Hanks buffer containing 0.5 mM ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) and 2% albumin (35). In the subsequent perfusion, 100 mL Hanks buffer containing 0.12% collagenase (type V, Boehringer, Mannheim, Germany) and 2 mM calcium chloride was employed. The perfusion times were 10 and 15 min with the first and second medium, respectively. The perfusion media were bubbled with carbogen gas (95% oxygen, 5% carbon dioxide) and warmed to 37°C before use. For dissociation of hepatocytes, the livers were shaken in Krebs-Henseleit buffer. The isolated cell suspension was centrifuged and after resuspension the same buffer was centrifuged at $80 \times g$ for 5 min. The yield from one liver was about 400×10^6 hepatocytes.

Subcellular fractionations. For subcellular fractionation, the cells were disrupted by sonication with a fine tip on a Heat System sonifier (Farmingdale, NY) using the lowest setting (36). The cell suspension in 0.25 M sucrose had a protein concentration of 30 mg/mL and was cooled in an ice-water bath. The sonication time was 1 min employing a 50% pulse. For preparation of the mitochondrial/lysosomal fraction, debris and nuclei were first removed by centrifugation at $480 \times g$ for 10 min. The supernatant was decanted and, after further centrifugation at $4300 \times g$ for 20 min, the pellet obtained was resuspended in 0.25 M sucrose and washed by centrifugation at $4300 \times g$ for 15 min. The washing procedure was repeated and the final mitochondrial/lysosomal pellet was resuspended in 0.25 M sucrose.

For the preparation of lysosomes, the $480 \times g$ supernatant was centrifuged at $48000 \times g$ for 12 min and the pellet was resuspended in 57% metrizamide adjusted to pH 7.3. The discontinuous gradient above the particle suspension was prepared according to Wattiaux *et al.* (37) and, after centrifugation at $100,000 \times g$ for 3 hr, the two bands on the top and bottom of the upper 20% metrizamide layer were collected and designated as lysosomes. The purified mitochondrial fraction, between the 57% and 37% metrizamide layers, was also collected.

For the preparation of microsomes, the suspension of disrupted cells was centrifuged at $10,000 \times g$ for 20 min and the supernatant was recentrifuged at $105,000 \times g$ for 60 min to obtain the microsomal pellet. The supernatant fraction was obtained by an additional centrifugation of the microsomal supernatant at $105,000 \times g$ for 2 hr.

The separation of microsomal and lysosomal membranes and luminal contents was achieved by sonication of the particle suspension for 1 min with a 50% pulse, followed by centrifugation at $105,000 \times g$ for 2 hr.

Marker enzyme activities were measured in mitochondria (cytochrome c oxidase), microsomes (NADPH-cytochrome c reductase), lysosomes (acid phosphatase) and plasma membranes (AMPase). The respective values were used to calculate the percentage contamination (on a protein basis) of the fractions isolated from hepatocytes (10,36,38). Mitochondria, lysosomes and plasma membranes contributed 0.5, 6 and 3%, respectively, of the total microsomal protein. The contamination of mitochondria ("lysosomes free") with microsomal protein, lysosomes and plasma membranes was 3, 3 and 1%, respectively. The lysosomal fraction contained 1, 9 and 2% mitochondria, microsomes and plasma membranes, respectively.

Microsomes prepared from hepatocytes were placed on a discontinuous sucrose gradient containing $MgCl_2$, as described earlier (36). Measurement of marker enzymes revealed that contamination with mitochondria, lysosomes and plasma membranes was 0.5, 4 and 1%, respectively. Thus, this gradient centrifugation removed a large portion of the plasma membrane contamination. The acid phosphatase activity not associated with organelles was also measured and it was found that 4% of the total activity of the homogenate was localized in the supernatant. Consequently, the majority of lysosomes in the homogenate were intact.

Protein was measured by the Biuret method, using bovine serum albumin as standard (39).

Preparation of liposomes. Twenty mg of egg lecithin (Lipid Products, South Nutfield, England) dissolved in chloroform/methanol (2:1, v/v) was mixed with 1 μmol [1-^{3}H]dolichol-19,[1-^{3}H]dolichyl-19 linoleate or 1,2-dipalmitoyl-*sn*-glycero-3-phospho[*methyl*-^{3}H]choline. The solvent was evaporated first under nitrogen and then *in vacuo*. The lipid residue was supplemented with 4.5 mL of 0.9% NaCl and sonicated for 10 min with a 50% pulse in an ice-water bath under nitrogen. This suspension was centrifuged at $105,000 \times g$ for 30 min before use. The liposomal suspension was prepared freshly each day.

Incubation. For incubations the cells from one liver were suspended in 40 mL Krebs-Henseleit buffer and supplemented with 2 mL of the liposomal suspension or 2 mL fatty acid/albumin complex (see below). The incubation was performed in a water bath at 37°C with continuous rotation. The mixture was also gassed with a continuous stream of carbogen. The incubation was terminated by cooling and centrifugation at $50 \times g$ for 4 min, after which the cells were washed four times with 20 mL of 0.9% NaCl solution. The final pellet was suspended in 0.25 M sucrose for subcellular fractionation.

Lipid extraction. Lipids from isolated subfractions were extracted with 20 vol of chloroform/methanol (2:1, v/v). Dolichol-23 and dolichyl-23 palmitate were added as internal standards. Partition was achieved with water, and the chloroform layer was then evaporated to dryness. The lipid residue was dissolved in methanol and applied to a Supelco C-18 cartridge (Bellefonte, PA). After washing with methanol, the dolichol and the dolichyl esters were eluted with hexane. After evaporation of the solvent, the lipids were dissolved in a small volume of hexane and analyzed by thin-layer chromatography (TLC) or by high-performance liquid chromatography (HPLC).

In order to isolate dolichyl-P, the subcellular fractions were subjected to alkaline hydrolysis by mixing 1 mL of the fraction with 2 mL methanol and 1 mL of 60% KOH. Dolichyl-23 phosphate was added as an internal standard. The mixture was heated at 80°C for 45 min. Lipids were subsequently extracted with chloroform/methanol/water (1:1:0.3, v/v/v). The extract was poured onto a column of DEAE-Sephadex (acetate form) and washed with the same solvent to elute uncharged molecules. Dolichyl-P was eluted with chloroform/methanol/water (1:1:0.3, v/v/v) containing 200 mM ammonium acetate. After partition, the chloroform phase was washed 3 times with theoretical upper phase in order to remove ammonium acetate. After evaporation of the solvent, dolichyl-P was dissolved in a solution (see below) containing 20 mM phosphoric acid and subjected to HPLC.

Chromatography. Separations by HPLC were done on a Hewlett-Packard (Palo Alto, CA) Hypersil ODS 3 μm

reversed phase column. A convex gradient (No. 1, Shimadzu solvent program, Kyoto, Japan) from the initial 2-propanol/methanol/water (40:60:5, v/v/v) in pump system A to 50% hexane/2-propanol (70:30, v/v) in pump system B was employed at a flow rate of 1.5 mL/min and with a program time of 25 min, and the absorbance of the eluate was monitored at 210 nm. Dolichols were well separated from dolichyl esters, which had longer retention times. Dolichyl-P was separated by HPLC as described earlier (16). The radioactivity in the dolichol, dolichyl-P and dolichyl ester fractions was determined by scintillation counting.

Chemicals. Dolichol-19 and dolichol-23 were isolated from autopsy specimens of human liver using reversed phase column chromatography. Dolichyl-19 linoleate was synthesized by esterification of dolichol-19 with the corresponding acyl chloride (40). For phosphorylation of dolichol-23, the method described by Danilov and Chojnacki was used (41). Labeling of dolichol-19 with tritium was achieved with NaB^3H_4 (14 Ci/mmol, New England Nuclear, Boston, MA), according to Keenan and Kruczek (42). The palmitic acid used in incubations was first complexed with albumin. Then, 75 μCi of [^{14}C]palmitic acid (57 mCi/mmol (The Radiochemical Centre, Amersham, England) and 7 mg of unlabeled palmitic acid were mixed, the pH adjusted to 9.5 and, after evaporation, the mixture was resuspended in 1 mL 0.9% NaCl (70°C, 20 min). Finally, the albumin complex was prepared by mixing 1 mL of the fatty acid solution with 1 mL 0.9% NaCl containing 200 mg fatty acid-free albumin at pH 7.4 (43). 1,2-Dipalmitoyl-*sn*-glycero-3-phospho[*methyl*-^{3}H]choline (72 Ci/mmol) was purchased from The Radiochemical Centre.

RESULTS

In agreement with previous studies, hepatocytes isolated by the collagenase method were found to effectively take up lipids from liposomes present in the incubation mixture (10). The chemical composition and surface charge of the liposomes had no great effect on the rate of uptake and, for this reason, egg lecithin was used as the major liposomal lipid in all experiments. The uptake of unilaminar vesicles was much more effective than was uptake of multilaminar liposomes. Care was taken to use liposomes that were prepared freshly by sonication. In control experiments the liposomes were added to hepatocytes. The hepatocytes were then fractionated and radioactivity associated with the microsomes was determined. Liposomes were also added to hepatocyte homogenates, which were fractionated and microsomes were analyzed. In neither case was a sizeable amount of radioactivity associated with the microsomal fraction, which excluded the possibility that cofractionation of liposomes had occurred.

Uptake of [^{3}H]dolichol. Hepatocytes take up measureable amounts of liposomal lipids within 30 min of incubation. In these uptake experiments, radioactive dolichol-19 was mixed with non-labeled dolichol to obtain a liposomal content of 2%. After 30 min of incubation, most of the dolichol had entered the cells and was associated with the microsomal fraction, whereas its concentration in the mitochondrial/lysosomal fraction was relatively low (Fig. 1A). The hepatocytes were then washed and the incubation was continued in the absence of liposomes. After 30 additional min of incubation, the microsomal dolichol concentration decreased rapidly, while the low level of radioactivity in the mitochondria/lysosomes was unchanged. After a 30-min incubation with liposomes, a portion of the dolichol in the microsomes became esterified with a fatty acid. It is known that the acyl-CoA:dolichol acyltransferase is localized in the endoplasmic reticulum (38) (Fig. 1B). The amount of dolichyl ester increased during the first 60 min of incubation, followed by a decrease during the next 30 min. In contrast to dolichol, dolichyl esters did not remain at the original low levels in lysosomes, but

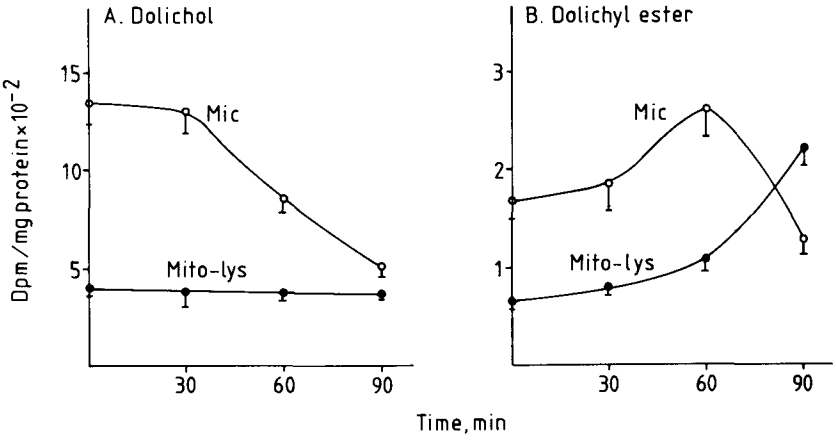

FIG. 1. Distribution and esterification of liposomal [^{3}H]dolichol in hepatocytes. Hepatocytes were incubated with liposomes containing [^{3}H]dolichol for 30 min. After this pulse labeling, the cells were recentrifuged, washed, and post-incubated for 90 min and the microsomes and mitochondrial/lysosomal fractions were prepared. Time 0 represents the start of the post-incubation period. The amounts of labeled dolichol (A) and dolichyl ester (B) were determined. The values are the means of 7 experiments; the vertical bars represent SEM.

increased continuously until after 90 min levels had increased 3-fold.

In some experiments, isolated microsomes were centrifuged on a $MgCl_2$-containing gradient, as described in Materials and Methods, which removed two-thirds of the plasma membrane contamination. There was no decrease in the [^{3}H]dolichol content in microsomes which had been purified in this manner, which shows that the label is not associated to any great extent with plasma membranes.

Uptake of [^{3}H]dolichyl linoleate. The previous experiment demonstrated that dolichyl ester, but not free dolichol, appears to be transported from the endoplasmic reticulum to the lysosomes (38). Therefore, experiments with dolichyl ester were also performed. When hepatocytes were incubated with liposomes containing [^{3}H]-dolichyl-19 linoleate, the amount of radioactive dolichyl ester recovered in microsomes increased rapidly (Fig. 2A). In the initial period, the level of the radioactive ester in the mitochondria/lysosomes was very low and an increase was observed only during the second half of the incubation period. A 5-min pulse gave relatively low labeling in the microsomes, and during the postincubation without liposomes radioactivity increased during the first 5 min followed by a decrease during the next 10 min (Fig. 2B). During the initial period of post-incubation, no significant increase in radioactivity associated with dolichyl ester could be observed in mitochondria/lysosomes and no free dolichol was detectable in the two organelle fractions studied.

Hepatocytes were also incubated with liposomes containing dolichyl esters for 30 min and relocation events were followed during a subsequent 100-min post-incubation period in the absence of liposomes. The microsomal labeling increased during the first 40 min of post-incubation, followed by a rapid and continuous decrease (Fig. 3A). The pattern of labeled polyisoprenoids in the supernatant was very similar to that found in microsomes. In the mitochondrial/lysosomal fraction, the initial low labeling increased rapidly and after 100 min was 5 times higher than the initial value. The initial low concentration of the labeled free dolichol in lysosomes increased rapidly during the entire incubation period (Fig. 3B). As concluded previously, the esterase activity specific for dolichyl esters is associated with lysosomes (44). The low levels of labeled free dolichol in the microsomes and supernatant did not change significantly.

After incubating hepatocytes for 80 min with liposomes containing [^{3}H]dolichyl-19 linoleate, the isolated microsomes and lysosomes were further treated to separate the membranes from the luminal contents (Table 1). In microsomes, both dolichyl ester and its hydrolysis product dolichol were present only in the membrane fraction. About half of both of these lipids was recovered in the lysosomal membrane and half in the luminal content. Clearly, dolichyl linoleate is transported from the endoplasmic reticulum, either through the cytoplasm or in a membrane-associated form, to the lysosomes, where it is recovered to a large extent in the soluble luminal compartment.

Phosphorylation of [^{3}H]dolichol. Hepatocyte microsomes contain a CTP-kinase, which is capable of phosphorylating dolichol under *in vitro* conditions (45). The liposomal system we used here is suitable for examining whether this kinase functions in hepatocytes *in vivo*. In our system only 2–3% of the total [^{3}H]dolichol appeared to be in phosphorylated form. Thus, this experiment suggests that in isolated hepatocytes exogenous dolichol is not phosphorylated to any significant extent.

Esterification with [^{3}H]palmitic acid. In preliminary experiments it was found that [^{3}H]palmitic acid presented to hepatocytes as a complex with albumin is effectively transferred to the cytoplasm and used in various types of enzymatic reactions. Hepatocytes were incubated with the [^{3}H]palmitic acid complex for 90 min, during which time labeled dolichyl ester accumulated both in the

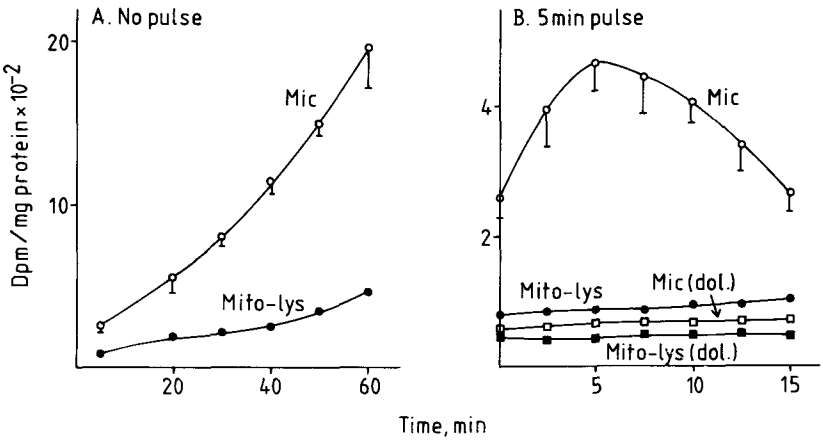

FIG. 2. Transfer of liposomal [^{3}H]dolichyl linoleate into hepatocytes. A. Liposomes containing dolichyl ester were present in the hepatocyte mixture throughout the entire incubation period. B. The hepatocyte suspension was incubated with the liposomes for 5 min followed by centrifugation and washing of the cells. The time periods given in the figure represent incubation times with the resuspended cells after the 5-min pulse labeling and subsequent washing. The values are the means of 9 experiments; the vertical bars represent SEM.

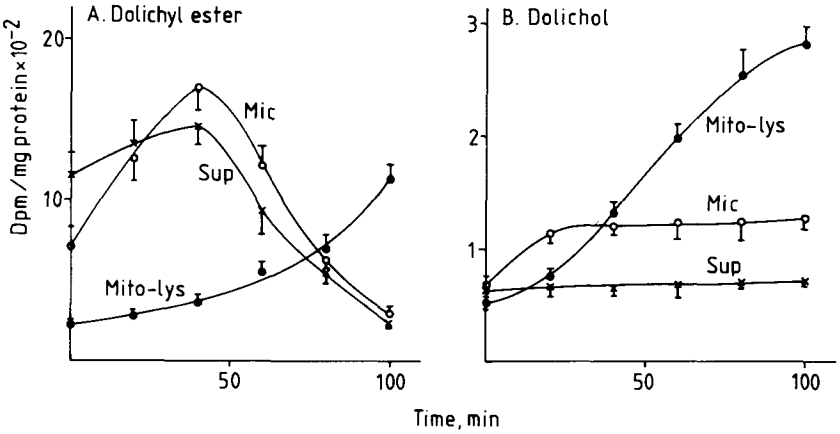

FIG. 3. Distribution and partial hydrolysis of liposomal [^{3}H]dolichyl linoleate in hepatocytes. Hepatocytes were incubated with liposomes containing [^{3}H]dolichyl linoleate for 30 min. After this pulse labeling, the cells were recentrifuged, washed, and post-incubated for 100 min. A. Distribution of [^{3}H]dolichyl ester in hepatocyte fractions. B. Appearance of nonesterified [^{3}H]dolichol in the isolated fractions. The values are the means of 7 experiments; the vertical bars represent SEM.

TABLE 1

Recovery of Liposomal [^{3}H]Dolichyl Linoleate in Subcellular Fractions from Hepatocytes[a]

	dpm/mg protein		
	Dolichyl ester	Dolichol	Total
Microsomes[b]			
Membrane	710 ± 75[c]	207 ± 25	917 ± 98
Luminal content	58 ± 6	34 ± 5	92 ± 11
Lysosomes[b]			
Membrane	776 ± 82	367 ± 39	1143 ± 139
Luminal content	689 ± 73	340 ± 31	1029 ± 109
Mitochondria[d]	386 ± 30	215 ± 20	601 ± 51
Supernatant	233 ± 24	100 ± 13	333 ± 38

[a]Hepatocytes were incubated with liposomes for 80 min.

[b]Membranes and luminal contents were prepared by sonication as described in Materials and Methods.

[c]Mean ± SD of 7 experiments.

[d]Mitochondria purified from the metrizamide gradient.

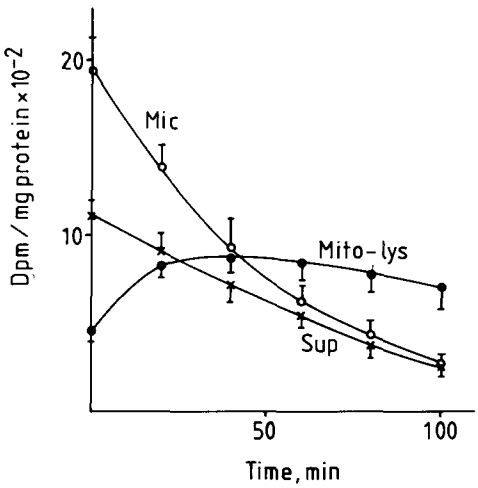

FIG. 4. Esterification of endogenous dolichol after incubation of hepatocytes with [^{3}H]palmitic acid-albumin complex. Hepatocytes were incubated with [^{3}H]palmitic acid for 90 min, followed by centrifugation and washing of the cells. The resuspended hepatocytes were post-incubated for 100 min. The values are the means of 6 experiments; the vertical bars represent SEM.

microsomes and in the supernatant (Fig. 4). In the following post-incubation period the concentrations of the radioactive lipid in both these fractions decreased rapidly during the initial 40 min and a concomitant increase in the mitochondrial/lysosomal fraction was observed. Thus, dolichyl ester synthesized within the hepatocyte exhibited a behavior similar to that of dolichyl ester supplied exogenously. Similar experiments were also performed using [^{3}H]mevalonate as biosynthetic precursor and the results obtained were similar to those described with [^{3}H]palmitic acid (data not shown).

Uptake of phosphatidyl[^{3}H]choline. The experiments with dolichol and dolichyl ester demonstrated preferential initial association of both lipids with the endoplasmic reticulum and subsequent transfer to the lysosomes. The question arose as to whether this behavior is typical for dolichols or is also seen with other lipids, such as phospholipids. Egg lecithin liposomes containing phosphatidyl[^{3}H]choline were therefore incubated with hepatocytes for 30 min (Fig. 5). The high level of labeled phospholipid in microsomes further increased during the first 40 min of post-incubation without liposomes followed by a continuous decrease in this fraction. The low levels of radioactivity in the mitochondrial-lysosomal fraction and in the supernatant were unchanged during the entire 100-min period (pre- and postincubation) studied. Thus, phosphatidylcholine supplied exogenously to the hepatocytes showed the same pattern of distribution as that described for dolichol.

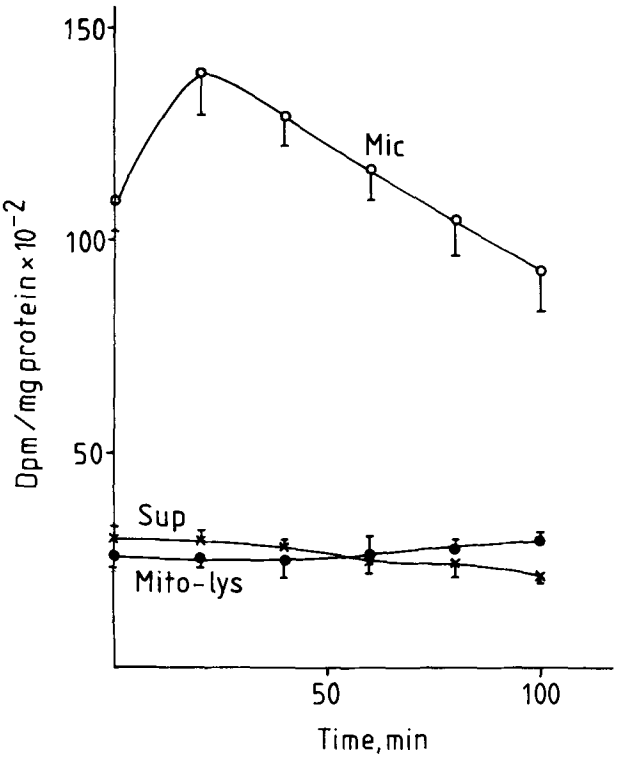

FIG. 5. Uptake of labeled phosphatidylcholine into hepatocytes. Hepatocytes were incubated for 30 min with liposomes containing 1,2-dipalmitoyl-*sn*-glycero-3-phospho[*methyl*-^{3}H]choline. The values show the distribution of label in the fractions during the post-incubation period. The values are the means of 6 experiments; the vertical bars represent SEM.

DISCUSSION

Hepatocytes isolated by EGTA-collagenase procedures are known to be of high quality, and a large number of metabolic and biosynthetic studies have been performed with these cells (46). Upon incubation with liposomes, the cells take up liposomal lipid components very efficiently and the rate of uptake greatly exceeds that observed with other types of cells (11). Interestingly, all lipid components of liposomes seem to be translocated into the cells, as observed here for free and esterified dolichol and phosphatidylcholine. This system seems suitable for studying the uptake of various lipids by hepatocytes since the process appears independent of the composition or the net charge of the lipids. A great number of experiments have demonstrated that during the 2–3 hr incubation, the cells appear to retain their normal metabolic capacity and that preincubation followed by washing allows efficient pulse labeling.

Previous investigations have shown that dolichol is taken up by cells, but the mechanism of its uptake has not been investigated in detail. The broad subcellular distribution of dolichol demonstrates that both the extracellularly supplied lipid and that synthesized in the cell are distributed effectively and do not remain only at one subcellular location. The transport mechanism for dolichol in the cell is not known, mainly because of the lack of suitable experimental systems. Our purpose was to supply the cell with highly labeled dolichol and to follow its appearance at various locations and to thereby obtain information on dolichol transport.

The experiments in this study have shown that the lipids appear first in the microsomes and that initially there is no uptake into mitochondria/lysosomes. Previous investigations had demonstrated that the mitochondrial dolichol content is low (47). Obviously, some incorporation of dolichol and dolichyl ester into mitochondria occurred in our system, but incorporation into lysosomes is much higher. Since the changes observed for the mitochondrial-lysosomal fraction reflect lysosomal changes, we used the combined fraction to deduce the events in lysosomes, as has previously been done (26). The uptake, evidently, is not receptor-mediated and does not involve the usual type of endocytosis. Liposomes rather appear to interact with the hepatocyte plasma membrane and with the individual lipid components in these structures not as liposomal membrane as such, but in micellar or carrier-bound form, to be transported to the endoplasmic reticulum.

One of the aims of our study was to find out in which form dolichol is transported within the cell. Upon incubation with dolichol, a small portion of the free dolichol became esterified in the endoplasmic reticulum and the dolichyl ester became transferred to the lysosomes within about 1–2 hr. These results are in agreement with previous investigations, where dolichol esterification and lysosomal transport were studied after metabolic labeling (38). Dolichol is esterified in the endoplasmic reticulum and the main purpose of this acylation appears to be to supply the lipid with a signal for further transport (48). Additional support for this idea of targeting was obtained when hepatocytes were incubated with the [^{3}H]palmitic acid—albumin complex. The early decrease in microsomal labeled dolichyl ester was paralleled by a concomitant increased labeling in lysosomes, again indicating that the ester is translocated.

In preincubated hepatocytes, microsomes had a high content of dolichyl ester, which, upon further incubation, decreased while the content in lysosomes increased. Evidence in support of the transport from the endoplasmic reticulum to lysosomes would be the demonstration of a precursor-product relationship, which is not possible at present.

Subfractionation of microsomal vesicles demonstrated that the translocated lipid is present mainly in the membrane. This may indicate that the lipid is transported *via* a membrane flow process, which is known to operate in the production of primary lysosomes, an organelle originating from the trans-Golgi reticulum. However, the presence of the dolichyl ester in the soluble cytoplasm during the post-incubation period suggests a cytoplasmic route of transfer. When microsomal esterification was monitored using [^{3}H]palmitic acid, dolichyl ester appeared rapidly in the cytoplasm, which suggests that the main mechanism operative in dolichyl ester transfer from the endoplasmic reticulum to lysosomes is a cytoplasmic transport and not a membrane flow.

Further transport of lysosomal luminal dolichol is of considerable interest. Lysosomal cholesterol is to a large extent transported to the bile (49) and it appears that such excretion occurs also with dolichol (15,16). There is experimental evidence that newly synthesized dolichol appears as free alcohol in the bile. According to this

mechanism, dolichol receives a fatty acid targeting signal in the endoplasmic reticulum, which is followed by its transport to the lysosomes. After hydrolysis by the specific lysosomal esterase, dolichol is translocated to the bile, either in unchanged or in metabolized form.

The exogenous dolichol recovered in microsomes is not phosphorylated to any significant extent, which indicates that the microsomal CTP-kinase does not produce dolichyl-P. This result is in agreement with previous investigations with hepatocytes from adult rats (50,51). In primary cell cultures where the mevalonate pathway was inhibited by compactin, only about 10% of the normal amount of dolichyl-P was found to be synthesized by phosphorylation of free dolichol (52). Pulse-labeling experiments with hepatocytes led also to the conclusion that dolichyl-P in these cells arises by *de novo* synthesis. In contrast, under conditions where the requirement for dolichyl-P is increased, such as during embryonic development and carcinogenesis, the CTP-kinase may play a role in dolichol phosphorylation (53,54). Consequently, it would be of interest in the future to conduct similar liposome/hepatocyte experiments with cells prepared from newborn animals or neoplastic tissue.

It is evident that liposomal lipids are transferred to the plasma membrane and, subsequently, to the cytoplasm by a mechanism which results in preferential incorporation into the endoplasmic reticulum. This pathway differs markedly from the receptor mechanism for the lysosomal uptake of low density lipoprotein, which has been studied in detail. Clearly, both mechanisms are operating for both phospholipids and neutral lipids. By monitoring these two processes, one may obtain detailed information about the distribution and excretion of lipids in hepatocytes.

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Effect of Ethanol on Platelet Phospholipase A_2

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Platelet aggregation is known to be inhibited by ethanol, and this has been suggested to be one of the attenuating effects of ethanol in cardiovascular disease. Recent studies have implicated an inhibition of phospholipase A_2 induced arachidonic acid release, since the production of prostanoids that are formed from arachidonic acid and are involved in the aggregation process has been shown to be diminished by ethanol. Phospholipase A_2 is found in platelets in both a cytosolic form, from where it may translocate to the plasma membrane to release arachidonic acid, and in a secretory form which is released extracellularly upon activation. In the present study, the effect of ethanol on the secretion of phospholipase A_2 and on its activity was determined. It was found that ethanol inhibited phospholipase A_2 secretion but not its activity. By contrast, the activity of the cytosolic form of phospholipase A_2 was inhibited by ethanol.
Lipids 27, 255-260 (1992).

There is considerable evidence that ethanol exerts an inhibitory effect on platelet aggregation (1-8), and it is possible that this could contribute to its attenuating effect in cardiovascular disease (reviewed in ref. 9). A number of studies have shown that ethanol inhibits the synthesis of prostanoids including thromboxane A_2, which is involved in the aggregation process (1-7). These findings would imply that ethanol reduces the level of arachidonic acid release. Indeed addition of exogenous arachidonic acid is known to alleviate the effect of ethanol (*e.g.*, see ref. 6).

The release of arachidonic acid may be due to the action of one or both of two enzymes, phospholipase C and phospholipase A_2. Phospholipase C activation would lead to the release of inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate then induces an increase in intracellular Ca^{2+}, and the diacylglycerol activates the regulatory protein kinase C. Arachidonic acid is then formed by the breakdown of diacylglycerol by diglyceride lipase. However, this pathway accounts for only a minor proportion of arachidonic acid release. The major source of arachidonic acid in platelets is phospholipids in which arachidonic acid resides in the *sn*-2 position and is released by the action of phospholipase A_2 (*e.g.*, see ref. 10,11). Recently, an inhibition by ethanol of the release of arachidonic acid has been demonstrated, and an effect on phospholipase A_2 was thereby implicated (12,13).

There is evidence for existence of more than one form of phospholipase A_2 in platelets (discussed in recent reviews, see ref. 14,15). Two types of phospholipase A_2 have been recognized, a cytosolic form of 90 kDa molecular weight (16-20) and a secretable, 14 kDa "type II" form (21-26; see also reviews 14,15). The cytosolic form can be translocated to the membrane surface as a result of an increase in intracellular Ca^{2+}, and it appears to be identical to a number of unknown membrane associated forms of the enzyme (27).

There is also evidence for arachidonoyl phospholipid species specificity of the cytosolic form of phospholipase A_2 in the platelet (16,19,28). However, platelet phospholipase A_2 can also hydrolyze other species (18,29-31), and if pre-labeled glycerol is used, all phosphatidylcholine (PC) species were found to be hydrolyzed to similar extents (29, 30). A lack of selectivity is also consistent with the observation that a partially purified rabbit platelet phospholipase A_2 did not show acyl-chain selectivity (32). Although in a recent study it was found that while the arachidonoyl PC species were hydrolyzed more rapidly than the linoleoyl PC species, in a mixture of the two species both were equally hydrolyzed (18). In spite of these latter observations, the release of the fatty acids from stimulated platelets, determined as mass changes in individual molecular species, does appear to show some arachidonoyl phospholipid species selectivity (16). An arachidonic acid selective phospholipase A_2 has recently been cloned (27). Taken together, above studies suggest that the phospholipase A_2, which is selective for arachidonate phospholipid species, may be compartmentalized in a manner which optimizes the release of arachidonic acid upon stimulation. This would fit in with the other observation that selectivity was best observed with minimal perturbation of the membranes (33). This may also imply that there may exist more than one cytosolic form of phospholipase A_2.

The secretable form of phospholipase A_2 is located in α-granules (21,25), as was shown by recent immunochemical-labelling studies (23). Detailed characteristics of the secretory phospholipase A_2, including substrate specificity, Ca^{2+} requirement, heat stability, and pH optimum, have been described (22,26). Studies in which phospholipase A_2 from rat and human platelets were compared have revealed that the level of secretion, or activity, of the rat phospholipase A_2 is much greater (22). At present there is no evidence for any fundamental differences between the rat and human phospholipase A_2 and it is entirely possible that observed differences in activity are due to the presence of an endogenous inhibitor.

While ethanol has been shown to inhibit the release of arachidonic acid from stimulated platelets (12,13), it is not known whether the effect is specific for the cytosolic form of the enzyme. To investigate the locus of ethanol action, the effects of ethanol on both forms of phospholipase A_2 were examined. Rat platelets were used since the secretory phospholipase A_2 is more readily obtained. The results revealed that ethanol inhibited the secretion of phospholipase A_2 but not its activity. By contrast, the cytosolic phospholipase A_2 was inhibited, although the effect was not specific to arachidonate.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats (100-200 g) were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). Thrombin, prostacyclin, indomethacin,

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Abbreviations: ATP, adenosine triphosphate; C_6-NBD-PC, *1*-palmitoyl-2-*N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino)hexanoylglycerophosphocholine; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HA, hexanoic acid; HEPES, *N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid].

and arachidonic acid were obtained from Sigma (St. Louis, MO); lipid standards and *1*-palmitoyl-*2*-*N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-glycerophosphocholine (C_6-NBD-PC) was from Avanti Polar Lipids (Birmingham, AL); 6-(*N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino) hexanoic acid) was from Molecular Probes (Eugene, OR). The radiolabeled lipids [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (206 Ci/mmol),*1*-stearoyl-*2*-[5,6,8,9,11,12,14,15-^{3}H]arachidonoyl-*sn*-glycero-3-phosphocholine (120 Ci/mmol) and 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (53.8 mCi/mmol) were from Amersham International (Arlington Height, IL). Solvents and other reagents were from Fisher Scientific (Pittsburg, PA).

Platelet preparation. Blood was taken by cardiac puncture from rats anesthetized by nembutol. The blood was anticoagulated with 0.38% (w/v) sodium citrate and centrifuged at 140 × *g* for 20 min at room temperature. The platelet-rich plasma was further centrifuged at 600 × *g* for 20 min and the pellet washed and resuspended in 10 mM (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) pH 7.4 buffer with 5 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 mM KCl, 145 mM NaCl, 10 mM dextrose, and 0.1 mM ethyleneglycol-*bis*(β-amino ethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA).

Phospholipase assay. For the release of arachidonic acid from activated platelets, [^{3}H]arachidonic acid was preincorporated by incubation of platelets with buffer containing 1 mg/mL fatty acid free albumin and [^{3}H]arachidonic acid (1 μCi/2 × 10^8 cells) for one hr in a shaking water bath; arachidonic acid not incorporated was removed by a centrifugation step. The cells were washed and finally resuspended in HEPES buffer pH 6.4 containing prostacyclin at 5–8 × 10^8 cells/mL. After incubation with thrombin (2U/10^6 cells) for 15 min, chloroform/methanol was added to the cells to stop the reaction and to extract lipids (34). The lipids from the chloroform phase (which included 0.01 mg/mL butylated hydroxytoluene to minimize oxidation) were then applied to a Silica Gel 60 plastic backed thin-layer chromatography (TLC) plate (EM Reagents, Cherry Hill, NJ) and separated using chloroform/methanol/acetic acid/H_2O (90:8:1:0.8, by vol). The phospholipids remained at the origin, and the location of arachidonic acid was verified by comparison with a standard. Corresponding regions were cut out, and radioactivity was determined using Budget-Solve scintillation fluid (Research Products Int., Mt. Prospect, IL).

The assay of the secreted phospholipase A_2 was performed using C_6-NBD-PC as the substrate, as previously described (35). Briefly, a suspension of C_6-NBD-PC (0.8 nmol) in buffer was added to platelets (0.4 mL, 2 × 10^8 cell/mL) or to the supernatant obtained from activated platelet suspensions (see above) in the presence of 1 mM Ca^{2+}. After 15 min, the reaction was terminated by addition of chloroform/methanol (34). The method depends on the release of the fluorescent fatty acid NBD-hexanoic acid from the *sn*-2 position and its recovery in the upper aqueous phase after the addition of chloroform/methanol, while the unhydrolyzed C_6-NBD-PC remains in the lower chloroform phase. The NBD-hexanoic acid was recovered from the upper aqueous phase and was easily quantified by virtue of its fluorescence relative to a standard.

Phospholipase A_2 activity of the particulate fraction of broken cell preparations was determined using vesicles consisting of natural phospholipids (100 μM) prepared from liver microsomes (36) / C_6-NBD-PC / *1*-stearoyl-*2*-[^{3}H]arachidonoyl-*sn*-glycerophosphocholine /*1*-palmitoyl-*2*-[^{14}C]oleoyl-*sn*-glycerophosphocholine (93:5:1:1, molar). The vesicles were made by mixing the components in a test tube, removal of the solvent by a stream of nitrogen, and addition of buffer. The suspension was then vortexed for 2 min to obtain a multilamellar vesicle suspension. The vesicles were added to the platelet phospholipase A_2, with 1 mM Ca^{2+}, and incubated at 37°C for 30 min. The reaction was terminated as described above. The release of radiolabeled arachidonic and oleic acid was determined by TLC of the lower chloroform phase. The release of NBD-hexanoic acid was determined as described above. TLC separations were carried out using Merck HPTLC plates (Darmstadt, Germany) as described previously (37). The positions of the phospholipids and fatty acids were verified by the use of lipid standards and were visualized by exposure to iodine vapor. The lipids were then scraped from the plate and radioactivity was counted as described above using an ^{3}H and ^{14}C dual window.

RESULTS

In previous studies with human platelets it was shown that the release of arachidonic acid is inhibited by ethanol (13,14). The experiments were repeated here using rat platelets, and essentially the same results were obtained (Fig. 1). The secretory phospholipase A_2 of rat platelets has been studied in some detail by others (22–26); its activity is somewhat higher than that of human platelets making it the system of choice for present study.

For study of the secretory phospholipase A_2 it was important to ascertain that cytosolic phospholipase A_2 was not able to hydrolyze the substrate. One way to separate the platelets from secreted phospholipase A_2 is by centrifugation. However, activated platelets are somewhat fragile. To be certain that membrane fragments from activated platelets would not remain in the supernatant with the secreted phospholipase A_2, high speed centrifugation would have been required to segregate the two. In addition, an unstimulated control would be difficult to obtain because centrifugation itself may cause stimulation. To avoid these complications, the phospholipase A_2 substrate C_6-NBD-PC was used. This substrate, while being rapidly taken up into the external bilayer leaflet of the plasma membrane, crosses the plasma membrane to the inner leaflet only very slowly (38), so it normally would not be hydrolyzed by cytosolic phospholipase A_2. While it is conceivable that flip-flop of phospholipids in the activated platelets may have allowed some access of the cytosolic phospholipase A_2 to C_6-NBD-PC, in a sonicated "particulate" preparation (from which the soluble or secreted phospholipase A_2 had been removed by centrifugation) while efficiently hydrolyzing natural PC substrates, only marginal activity against C_6-NBD-PC was noted (see below). Therefore the contribution of cytosolic phospholipase A_2 (in the inner leaflet) to the hydrolysis of any C_6-NBD-PC which had crossed to the cytosolic side should have been negligible. Separation of the stimulated platelets from the secreted phospholipase A_2 was therefore not obligatory. The assay also offers other advantages as previously detailed (39).

The optimal concentration of thrombin required for the phospholipase A_2 secretion is shown in Figure 2. It was

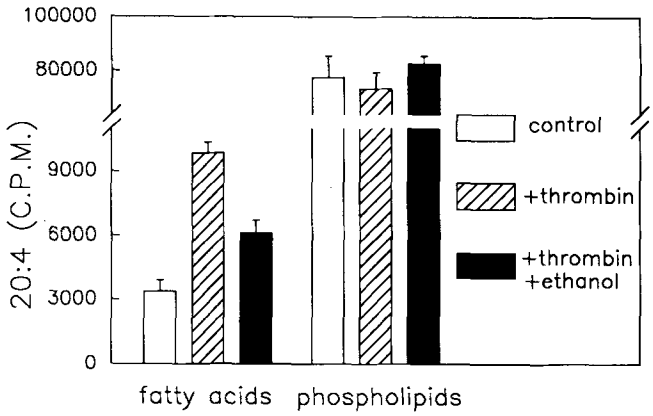

FIG. 1. Effect of ethanol (100 mM) on thrombin (0.2 U/mL) stimulated release of arachidonic acid from rat platelet membrane phospholipids. Results are expressed as the arachidonate released in the form of free fatty acid and the arachidonate remaining esterified to the platelet phospholipids (CPM, mean of ⩾ 3 experiments ± SD, details are as described in Materials and Methods).

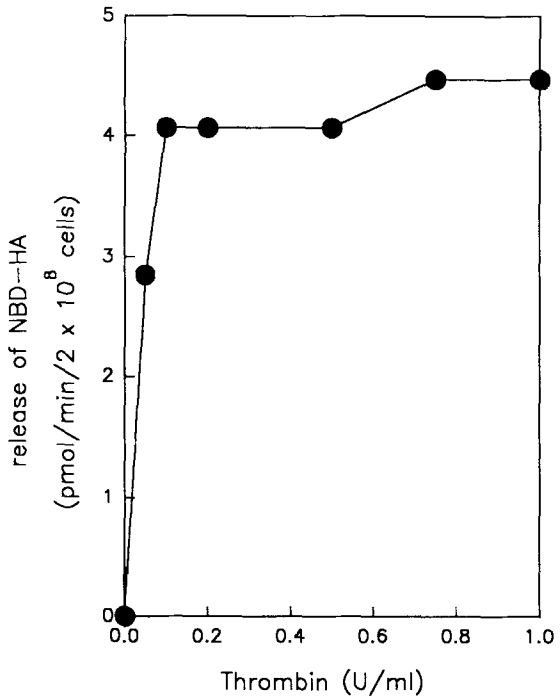

FIG. 2. The thrombin concentration dependence of the secreted phospholipase A_2, assessed by the hydrolysis of C_6-NBD-PC (details as described in Materials and Methods).

found that phospholipase A_2 activity increased with thrombin concentration to ~ 2 U/mL after which it reached a maximum. The secretory phospholipase A_2 could also be obtained by sonication in the absence of thrombin or Ca^{2+}.

In order to confirm that the phospholipase A_2 activity detected with activated platelets using C_6-NBD-PC was soluble, the cells were pelleted by centrifugation (600 × *g* for 10 min) and the supernatant and the pellet was examined for phospholipase A_2 activity. It was found that phospholipase A_2 was indeed released from the cells since activity was recovered in the supernatant (see Fig. 3). The phospholipase A_2 release, after thrombin stimulation, was extremely rapid, occurring in seconds. The phospholipase A_2 release showed no further increase, as demonstrated by recovering the released phospholipase A_2 at different time points after activation (Fig. 3). There was a slight apparent decrease of activity with time after the initial secretion, which may have been due to some re-incorporation of NBD-hexanoic acid (NBD-HA) (released by the phospholipase A_2) into other membrane phospholipids prior to the centrifugation of the cells. It should be noted that the activity was higher after sedimentation than in cell suspensions. Thus activation by the centrifugation procedure may have activated resilient platelets that had not activated by thrombin alone and required the extra stimulus, or the centrifugation released trapped phospholipase A_2. It is also not possible to exclude that some cytosolic phospholipase A_2 may have been released. However, if C_6-NBD-PC is a poor substrate for cytosolic phospholipase A_2, as evidence suggests, and considering that the total level of activity of this phospholipase A_2 was much lower than that of the secreted form, this should not have been a significant factor. To confirm that the released phospholipase A_2 was soluble, the supernatant was also centrifuged at 100,000× *g* for 60 min. Examination of the supernatant still revealed the presence of phospholipase A_2 (7.8 pmol NBD-HA/min/2 × 10^6 cell, similar to the activity obtained after low speed centrifugation) showing that it was the secreted form. The production of NBD-hexanoic acid, indicative of phospholipase A_2 activity was confirmed using TLC and respective standards. Although the centrifugation procedure was effective for measuring the time course of phospholipase A_2 release and to demonstrate that the released phospholipase A_2 was soluble, problems in using centrifugation with platelets for studying the secreted phospholipase A_2 point to the advantage of using the C_6-NBD-PC substrate.

To explore the effect of ethanol on the phospholipase A_2 secretion, thrombin activated platelets were examined by addition of ethanol prior to stimulation. The phospholipase A_2 activity and/or amount of phospholipase A_2 released was reduced as shown in Figure 4.

The effect of ethanol on the secretory phospholipase A_2 (*after* activation) was also investigated to determine if the amount of phospholipase A_2 secreted, or its activity, was affected by ethanol. Platelets were subjected to 5 × 15 sec bursts of ultrasonication (with 15 sec pauses) using a Fisher Sonicator at 50% full power with a microtip probe, followed by ultracentrifugation at 100,000 × *g* for one hr and separation into soluble and particulate fractions. To the soluble and particulate fractions, respectively, was added a PC vesicle suspension containing labeled arachidonoyl, oleoyl and NBD-hexanoyl PC (as described in Materials and Methods). Hydrolysis of the three substrates was simultaneously determined and is shown in Figure 5. The results reveal that there was only a marginal effect of ethanol on the secretory phospholipase A_2 *activity* for all three substrates, as distinct from an inhibition of *secretion* of phospholipase A_2. The rate of hydrolysis of phospholipase A_2 in the particulate fraction was much lower than that for the secretory phospholipase A_2. This may have been due to a lesser amount of phospholipase A_2, a lower activity, or to difficulties in sub-

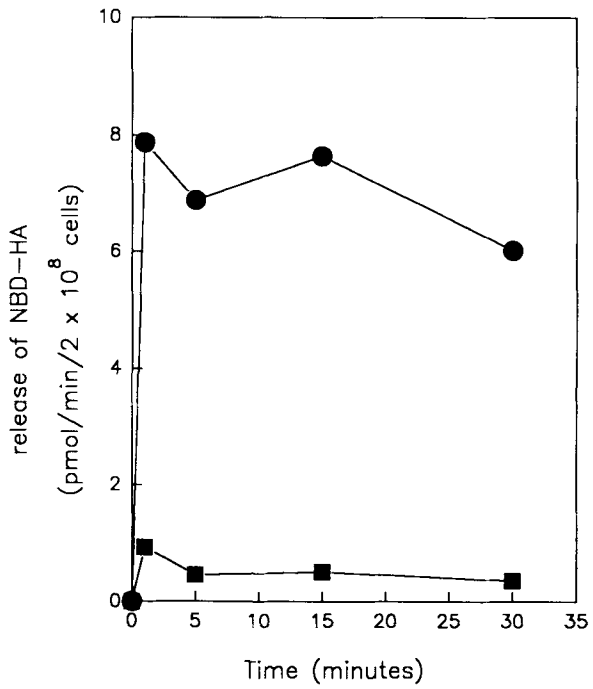

FIG. 3. The phospholipase A_2 activity (using the substrate C_6-NBD-PC) recovered in the supernatant [filled circle] from platelets stimulated using thrombin (0.2 U/mL and 1mM Ca^{2+}) at different times of stimulation separated as described in Materials and Methods.

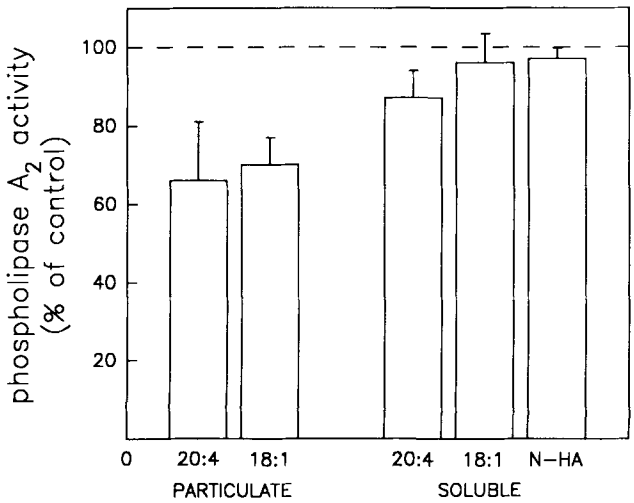

FIG. 5. Effect of ethanol (100 mM) on the activity of membrane associated phospholipase A_2 and on the phospholipase A_2 secreted from thrombin stimulated platelets. The results are expressed as the activity of phospholipase A_2 (in the presence of 100 mM ethanol) as a percentage of the activity in the absence of ethanol (soluble: 2.5ϱmol NBD-HA, 3.8ϱmol 18:1 and 3.2ϱmol 20:4/min/10^8 cells; particulate: 0.14ϱmol 18:1 and 0.12ϱmol 20:4/min/10^8 cells). The phospholipase A_2 activity was determined against PC multilamellar vesicles consisting of natural phospholipids (100 μM, prepared from liver microsomes, C_6-NBD-PC,*1*-stearoyl-*2*-[^{3}H]arachidonoyl-glycerophosphocholine and *1*-palmitoyl-*2*-[^{14}C]oleoyl-glycerophosphocholine (93:5:1:1 molar, details as described in Materials and Methods). The results are the mean of three determinations from one of two replicate experiments.

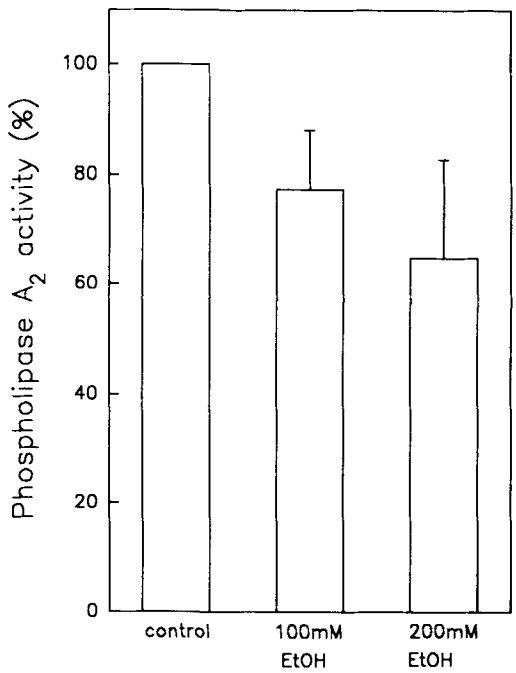

FIG 4. Effect of ethanol on the thrombin induced secretion phospholipase A_2, assessed by the hydrolysis of C_6-NBD-PC. The results are expressed as the activity of phospholipase A_2 as a percentage of the control (4.25ϱmol NBD-HA/min/10^8 cells). The activities in the presence of ethanol were significantly different from the control at the 0.05% level according to the paired t-test; the results are the mean of three experiments ± SD (details as described in Materials and Methods).

strate accessibility by the cytosolic phospholipase A_2. Alternatively, this could have been due to the cytosolic phospholipase A_2 being released in the course of the sonication process, although the level of phospholipase A_2 obtained by sonication and thrombin were similar. In addition, the rate of C_6-NBD-PC hydrolysis in the particulate fraction was too low to be accurately measured, possibly due to a preference of the cytosolic phospholipase A_2 for the natural PC substrates. The important finding was that while ethanol did not affect the secretory phospholipase A_2 activity, ethanol inhibited the hydrolysis of arachidonoyl- and oleoyl-PC in the particulate fraction as shown in Figure 5.

DISCUSSION

In previous studies it had been shown that ethanol inhibited the release of arachidonic acid from human platelets (13,14). To determine which of the two forms of phospholipase A_2 might be responsible, the effects of ethanol on both the secretory and cytosolic forms were examined. It was found that ethanol inhibited secretion of the secretory form of phospholipase A_2 but not its activity. By contrast, the cytosolic phospholipase A_2 was inhibited by ethanol.

We were able to demonstrate that rat platelets secrete phospholipase A_2 upon thrombin induced stimulation, confirming previous observations (21–26), but using C_6-NBD-PC as substrate. The use of C_6-NBD-PC in the investigation of platelet phospholipase A_2 is a novel and useful approach offering a convenient assay system, how-

ever, for phospholipid specificity studies, conventional methods with radiolabeled lipids are still advantageous. Next, it was demonstrated that ethanol inhibits the release of arachidonic acid from activated rat platelets as was previously demonstrated for human platelets (13, 14). The release of arachidonate resulting from platelet activation is normally considered to arise from the action of a cytosolic phospholipase A_2 after being bound to the plasma membrane. It should be noted, however, that the secreted phospholipase A_2 could potentially act on the external leaflet of the platelet membrane and could induce the release of arachidonate, a point worthy of future study.

In the present study, inhibition by ethanol of the secretion of phospholipase A_2 was observed. Ethanol is known to inhibit the secretion of 5-hydroxytryptamine and adenosine triphosphate (ATP) in stimulated human platelets (13,14), and it is therefore reasonable to explain the inhibition of phospholipase A_2 secretion by such a mechanism. Prior to the present study involvement of the secretory phospholipase A_2 had not been established. The mechanism whereby ethanol inhibits the secretion process is unknown; however, it has been shown that thrombin induced secretory granule centralization and fusion is inhibited by ethanol (14,40). Some experiments were performed to determine the secreted phospholipase A_2 from human platelets (results not shown); however, it was found that only very small amounts were released. This is consistent with the results of other studies (22). This shows that the rat platelet is a more useful model to determine the effects of ethanol on the secreted form of phospholipase A_2.

When the activity of the cytosolic phospholipase A_2 was examined, ethanol was found to inhibit its activity in terms of the release of *sn*-2 oleic or arachidonic acid from phosphatidylcholine. This contrasted with the lack of an effect of ethanol on the secretory form of the enzyme. The inhibition of the cytosolic form of phospholipase A_2 could occur either directly by action of ethanol on the enzyme itself or indirectly by acting on the various other regulatory mechanisms that occur in the platelet. There are relatively few studies on the effect of alcohol on phospholipase A_2. In one study concerned with venom phospholipase A_2, a slight stimulatory effect rather than an inhibition was found; however, the form of the enzyme differs greatly from the cytosolic phospholipase A_2 (41). Notwithstanding possible differences between the secreted phospholipase A_2 of rat and human platelets, there is no evidence to suggest that the membrane associated cytosolic forms of phospholipase A_2 differ to any extent so that an inhibition of human platelet membrane associated phospholipase A_2 by ethanol might be expected.

At present we can only speculate as to the mechanism of the inhibition by ethanol of membrane associated phospholipase A_2. As a pointer to further studies, it is worth noting that there is increasing evidence for a G-protein and/or protein kinase C involvement with (cytosolic/membrane associated) phospholipase A_2 in other tissues (42,43) as well as in platelets (44–46). It is quite possible that ethanol acts at the level of these proteins, as demonstrated, for example, for a G-protein association with phospholipase C in turkey erythrocytes (47). Other possibilities include protein kinase C which may regulate platelet phospholipase A_2 *via* phosphorylation, again probably *via* a G-protein (44,48,49).

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The technical assistance of D. Dicenzo, T. Williams, A. Kisielewski and S. Gross are gratefully acknowledged. This work was supported by U.S. Public Health Service Grants AA 08022, 07215, 07309 and 07186 and a grant from the Alcoholic Beverage Medical Research Foundation.

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The Dual Effect of Oxidation on Lipid Bilayer Structure

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Sphingomyelin membranes were prepared with different levels of oxidative damage caused by *tert*-butyl hydroperoxide (TBH). Temperature-induced changes in membrane hydrocarbon chain packing (phase transitions) were monitored using infrared spectroscopy. Lipid phase transition characteristics were evaluated from thermodynamic parameters fitted to the experimental transition curve data. At temperatures below the lipid phase transition T_c, hydrocarbon chains pack in an ordered state whereas above the T_c the hydrocarbon chains pack in a disordered state. Compared to the non-oxidized control, the packing of the hydrocarbon chains of mildly oxidized sphingomyelin (<10 nmol TBH/mg lipid) was no different at all temperatures below the T_c, and was more ordered above the T_c. The hydrocarbon chains of strongly oxidized sphingomyelin (>10 nmol TBH/mg lipid) were more disordered at temperatures above and below the T_c compared to the control samples. These results suggest that lipid oxidation has a dual effect on lipid order. A more ordered or disordered state may result depending on the degree of oxidation and the state of lipid order prior to oxidation. These results could be important for explaining the structural changes in oxidized membranes high in sphingomyelin such as those found in the ocular lens and liver plasma membranes.
Lipids 27, 261-265 (1992).

Lipid oxidation has been implicated in a number of pathological processes (1-4, and references therein) and is believed to alter protein structure and membrane function (5-10). The function of biological membranes is directly affected by the conformation and packing of the lipid components (11). An increase in the passive permeability of membranes has been attributed to conformational changes caused by lipid oxidation (12-17). Several studies have demonstrated that lipid oxidation orders the lipid matrix (18-27).

Of particular interest is the role of lipid oxidation in the process of cataractogenesis in the ocular lens (1). We have shown that low levels of oxidative damage induced by *tert*-butyl hydroperoxide (TBH) cause sphingomyelin, the major phospholipid of the lens membrane, to become more disordered (28). Both the headgroup and acyl chain regions of the bilayer were deranged (28). These results appear to be contrary to those reported for lipids that are fluid at physiological temperature (18-27). The purpose of this report is to determine the cause(s) of this disparity and to develop a general structural model that could explain previous and current results.

METHODS

Liposome preparation and oxidation. Liposome bilayer vesicles were prepared by dispersing sphingomyelin in an aqueous buffer consisting of 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid and 100 mM KCl at pH 7.4. Sphingomyelin from bovine brain (99%) and all reagents were obtained from Sigma Chemical Co. (St. Louis, MO), and were used without further purification. Samples, each having approximate concentrations of 1 mg lipid per mL of buffer, were treated with TBH at 2-35 nmol/mg lipid. TBH-treated liposomes are hereafter referred to as "treated samples". The sphingomyelin from these samples was partitioned into chloroform at low temperature in an argon atmosphere to prevent auto-oxidation of hydrocarbon chains as described previously (28). This protocol removes any water-soluble secondary oxidation products in the aqueous phase. The extracted lipid was then dried under a stream of argon. Absorption spectra indicated that at least 99% of the lipid partitioned into the lower $CHCl_3$ phase.

Instrumentation and preparation of samples for spectroscopy. Liposomes were prepared by dispersing dried lipid samples, as described above, in a buffer at 150 mg/mL lipid. Thin films of samples were prepared by sandwiching 3 μL of sample placed between 13-mm diameter AgCl plates with a 0.015-mm spacer. Samples were warmed to 70°C for one hour to assure complete hydration. The sample was cooled from 70 to 0°C; then heated from 0 to 70°C. Temperature was changed at a rate of 0.1°C per min to avoid hysteresis and was controlled within ±0.1°C. Hysteresis, in this case is the lagging behind of transition properties, such as fluidity, when a sample is heated or cooled too quickly. The sample was allowed to equilibrate at a given temperature for 5 min before a spectral measurement was made. Infrared spectra were measured every 5°C which took about 2 min per spectrum. The spectra were acquired on a Nicolet 7199C Fourier transform infrared spectrometer (Cincinnati, OH), equipped with a triglycine sulfate detector. One hundred interferograms were co-added and apodized with a triangular function. The resulting effective spectral resolution was 2 cm^{-1}. The frequency of the lipid acyl chain CH_2 symmetric stretching band was measured with a precision of ±0.06 cm^{-1}. This precision was calculated from a set of independent measurements from four different preparations of the same sample.

Statistical evaluation of the lipid phase transition. It is difficult to measure and assess the accuracy of parameters such as relative cooperativity by visual inspection of lipid phase transitions curves. To circumvent these problems, a non-linear regression analysis was used to evaluate the parameters P_1, P_2, P_3 and P_4 from experimental phase transition data pairs (vibrational frequency in wavenumbers, temperature in K). The errors in the fitted parameters are usually greater than the precision by which the frequencies are measured. The function used in the fitting algorithm given below has been used previously (29):

$$\text{frequency} = P_1 + P_2/[1 + (P_3/T)^{P_4}] \qquad [1]$$

P_1 is the minimum wavenumber for the phase transition and represents the most ordered state of the transition.

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Abbreviations: TBH, *tert*-butyl hydroperoxide; UV, ultraviolet.

P_2, the magnitude of the phase transition, is the net change in wavenumber and represents the change in the number of *trans*-to-*gauche* rotomers. P_3 is the transition temperature and indicates the temperature at which half of the population of lipid molecules has undergone the phase change. P_4 is the relative cooperativity and describes the degree by which one lipid affects the state of order of adjacent lipids.

The enthalpies of the lipid phase transitions were calculated from Arrhenius plots (ln% *gauche* rotomers *vs* 1/T) as described previously (29).

The maximum frequency is a parameter that quantifies the maximum disorder of the hydrocarbon chains. It is calculated as the sum of P_1 and P_2, and represents the maximum frequency at an infinitely high temperature (see equation above).

RESULTS AND DISCUSSION

Fourier transform infrared spectroscopy was used to measure structural changes which resulted from oxidative damage induced by TBH treatment. Infrared spectroscopic techniques are not troubled by the limitations of spin probe techniques used to study oxidative damage on lipid structure (18-24,26,27). These limitations include: the influence of the probe on adjacent lipid molecules (30), the uncertainty of the location of the probe (31), the ineffectiveness of the probe at higher concentrations of cholesterol (32), such as those found in lens membranes (33), and the chemical modification of the probe (34). At best, spin probe studies only infer the order of lipid membranes and are not a direct measure of lipid conformation, environment and bilayer packing.

At lower temperatures, lipid hydrocarbon chains are usually in an ordered phase (gel phase), the fatty acyl carbons are in an all *trans* conformation, and van der Waals interactions and bilayer packing are maximized. At higher temperatures, lipids often form a disordered phase (liquid crystalline phase) where a number of C-C *gauche* rotomers are introduced diminishing van der Waals interactions between hydrocarbon chains (35,36). Thus upon increasing temperature, many lipid bilayer membranes undergo a change from an ordered to a disordered phase. The frequency of the CH_2 stretching band near 2850 cm^{-1} increases as the disorder and number of gauche rotomers increase. This band was used in this study to monitor the order of the lipid hydrocarbon chains as a function of temperature. From temperature *vs* lipid order curves, phase transition parameters were determined.

Figure 1 shows temperature-induced phase transitions for a suspension of sphingomyelin vesicles prior to (lower curves) and after (upper curves) oxidation. The gel-to-liquid crystalline phase transition was observed at about 36°C for the untreated lipid. The broad phase transition curves corresponding to samples treated with concentrations greater than 10 nmol TBH/mg lipid indicated the absence of a sharp phase transition over the temperature range measured. For this reason, above 10 nmol TBH/mg lipid, the T_c could not be assessed. However, other parameters were evaluated independently of the T_c. Below 8 nmol TBH/mg lipid, a linear increase in oxidative damage (28) caused no significant change in the transition temperature (Fig. 2A).

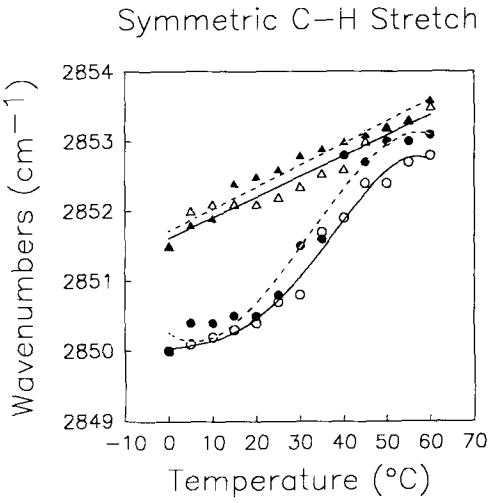


FIG. 1. Change in frequency of the CH_2 symmetric stretching band with temperature for sphingomyelin membranes: before (circles), and after (triangles) treatment with 20 nmol TBH/mg lipid. Closed symbols and dashed lines are for the cooling curves. Open symbols are for the heating curves. Higher wavenumber values indicate a more disordered membrane.

We have shown that the conjugated diene content increases linearly, and quantitatively with TBH concentrations between 0 and 50 nmol TBH/mg sphingomyelin (28). Precise quantification of the conjugated diene content measured by ultraviolet (UV) absorption at 241 nm is impeded by spectral interference due to bands corresponding to isolated double bonds and ketones. Consequently, the initial number of conjugated dienes is an approximation and is about 1 conjugated diene in 50 moles sphingomyelin. We have determined precisely that treatment of sphingomyelin with TBH (from 0 to 30 nmol TBH/mg sphingomyelin) causes a linear increase in the content of conjugated dienes. Four moles of conjugated dienes are formed per 50 moles of sphingomyelin when 1 mg of sphingomyelin is treated with 30 nmol TBH.

Mild oxidation, <10 nmol TBH/mg lipid. It is evident from Figure 2B that for concentrations below 10 nmol TBH/mg sphingomyelin, increasing lipid oxidation decreases the maximum C-H stretching frequency, which implies a decrease of acyl chain *gauche* rotomers, a measure of lipid disorder. Thus when sphingomyelin is in the disordered phase, oxidation levels below 10 nmol TBH/mg lipid order the membrane. These results are in accordance with those obtained using invasive probe techniques (18-27). This ordering effect with mild levels of oxidation is shown in Step 2b of Scheme 1. It is possible, for example, that at mild levels of lipid oxidation (<10 nmol TBH/mg lipid) *trans* double bonds are formed which may rigidify the bilayer (26,37). One would expect the transition enthalpy of the system to increase due to this type of stabilization. The transition enthalpy in fact remains constant with mild oxidation (Fig. 3). This disparity may be explained by a decrease in the magnitude of the phase transition (Fig. 2C). The decrease indicates that a smaller percentage of lipids are involved in the transition equilibrium 3 compared to that in equilibrium 1 (Scheme 1). The lack of significant change in the cooperativity (Fig. 2D) below 10 nmol TBH/mg lipid indicates

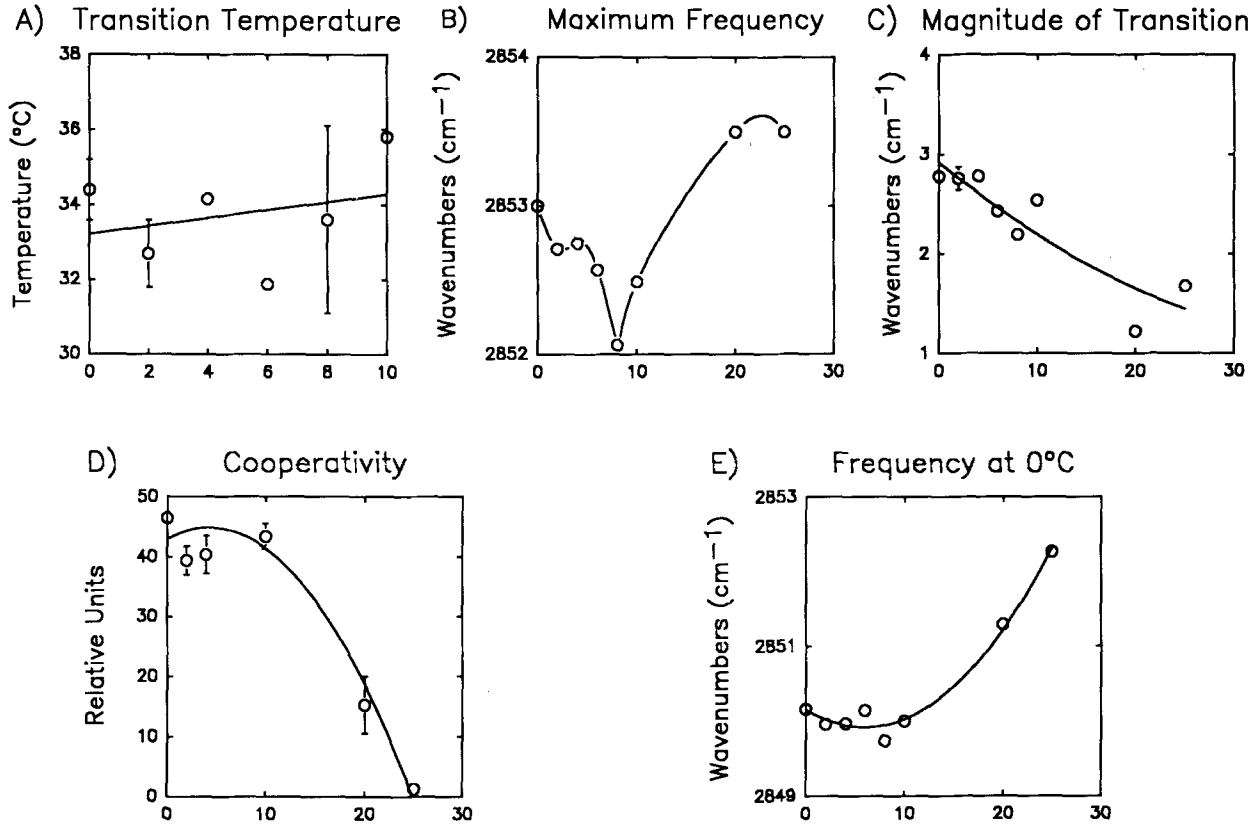
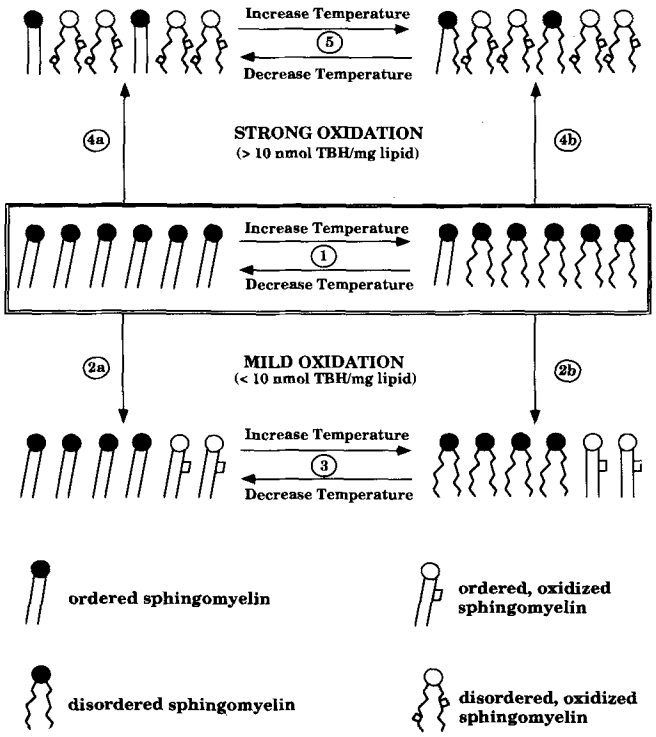


FIG. 2. The thermodynamic parameters were determined using non-linear regression analysis (see Methods) applied to phase transition curves such as those shown in Figure 1. X-axis is the concentration of TBH in nmol/mg lipid. Error bars are given for all data points unless the error is smaller than the symbol. In Figure 2A, the error bar for the data point corresponding to an abscissa value of 10 is ±7.



SCHEME 1

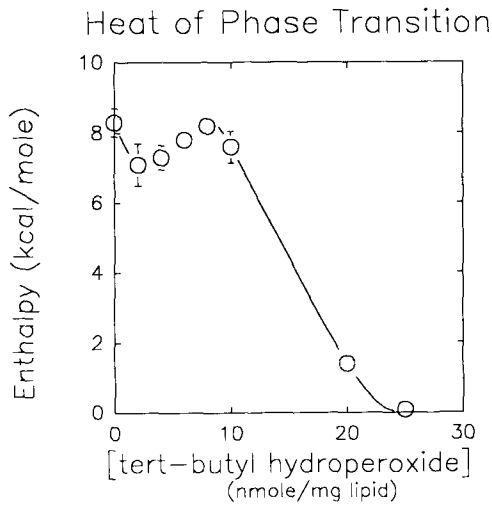


FIG. 3. The enthalpy of the lipid phase transitions was calculated from an Arrhenius plot (ln % *gauche* rotomers *vs* 1/T).

that the ordered oxidized lipids are laterally phase separated from the fluid lipids. The frequency at 0°C (Fig. 2E) does not change, indicating that mild oxidation has no effect on sphingomyelin in the ordered state (Scheme 1, Step 2a).

Strong oxidation, >10 nmol TBH/mg lipid. A small change in the maximum frequency (Fig. 2B) with strong oxidation indicates that at this level of lipid oxidation, packing of the hydrocarbon chains in the disordered phase is affected only slightly by oxidation. This is represented in Scheme 1, Step 4b. It should be noted that oxidation may have a more dramatic effect on the headgroup or interface region, packing, and/or may affect the dynamics of lipid motion. All of these parameters are not detected by merely measuring hydrocarbon chain structure. The observed increase in the frequency at 0°C (Fig. 2E) for high levels of oxidation may be considered as a disordering of the bilayer (Scheme 1, Step 4a). This disordering of the ordered phase at higher levels of oxidation has not been reported previously and may be caused by the disruption of van der Waals interactions by the introduction of hydrophilic peroxy groups into the hydrophobic lipid acyl chain environment. It is apparent that this increase in the level of disorder can overcome the ordering effect of *trans* bond formation observed at lower levels of oxidation. This is a unique finding and implies that natural membranes that are highly ordered, such as the ocular lens membranes, would become more fluid with increasing oxidation. We have observed this in a preliminary study of cataractous lens membranes (38) where lipids have been shown to contain a higher degree of oxidation than normal lens lipids (1). Oxidized disordered lipids in an ordered lipid matrix would be expected to increase the passive permeability of these membranes (39). An increase in passive permeability is observed during cataractogenesis (40-42).

A decrease in cooperativity (Fig. 2D) indicates that below the transition temperature, the unoxidized lipid is not laterally phase separated but dispersed in a fluid lipid matrix (Scheme 1, equilibrium 5). A decrease in the enthalpy (Fig. 3) and magnitude (Fig. 2C) of the phase transition indicates that fewer lipid molecules undergo an order to disorder phase change (Scheme 1, equilibrium 5) as compared to the mildly oxidized membranes (Scheme 1, equilibrium 3) and untreated membranes (Scheme 1, equilibrium 1). When membranes are highly oxidized and close to their maximum state of disorder, a slight disordering of the hydrocarbon chains is observed (Scheme 1, Step 4b).

Dimyristoyl phosphatidylcholine was used as a control since this phospholipid has no double bonds and is not oxidized with TBH. None of the thermodynamic parameters for dimyristoyl phosphatidylcholine were influenced by TBH treatment (Table 1). Thus all of the effects of TBH on sphingomyelin appear to be due to oxidative perturbation.

We have shown that lipid oxidation has a dual effect on lipid order in that it may result in a more ordered or a more disordered state depending on the degree of oxidation and the state of lipid order prior to oxidation. These studies will serve as a foundation to explain structural changes caused by oxidative damage to native membranes that are high in sphingomyelin, such as lens and liver plasma membranes.

TABLE 1

Thermodynamic Parameters for the Order to Disorder Phase Transition of Dimyristoyl Phosphatidylcholine Membranes Before and After Treatment with Oxidant

	Untreated	Treated with oxidant (20 nmol TBH/mg lipid)
Minimum frequency (cm^{-1})	2849.9 ± 0.2	2850.5 ± 0.1
Maximum frequency (cm^{-1})	2853.2 ± 0.3	2853.5 ± 0.2
Phase transition magnitude (cm^{-1})	3.3 ± 0.2	3.0 ± 0.2
Phase transition temperature (°C)	31 ± 0.9	29 ± 0.7
Cooperativity	119 ± 65	122 ± 46

ACKNOWLEDGMENTS

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Decreased Lecithin:Cholesterol Acyltransferase Activity in the Plasma of Hypercholesterolemic Pigs

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Lecithin:cholesterol acyltransferase (LCAT) activity levels were determined, as function of plasma total cholesterol (TC) in 13 normocholesterolemic (TC < 85 mg/dL) and in 28 hypercholesterolemic (TC > 98 mg/dL) pigs. The normocholesterolemic group consisted of pigs that carried apo-B allelic genes other than *Lpb*^{5} and or *Lpb*^{8}. The hypercholesterolemic group consisted of *Lpb*$^{5/x}$ and *Lpb*$^{5/8}$ heterozygous and *Lpb*$^{5/5}$ homozygous animals. The data reported in this study show that the LCAT activity in the plasma of hypercholesterolemic (HC) pigs (79 ± 43 units) was significantly lower ($p < 0.0005$) compared to the normocholesterolemic controls (175 ± 45 units). Furthermore, LCAT activity was positively correlated with TC in the normocholesterolemic group ($r = +0.54$; $p < 0.05$), whereas it was negatively correlated with TC in the hypercholesterolemic group ($r = -0.73$; $p < 0.001$). Additional data obtained from incubation experiments suggest that the lower LCAT activity in hypercholesterolemic pigs may be due, at least in part, to inhibition of LCAT activity by components found in the lipoprotein-deficient fractions of the plasma of hypercholesterolemic pigs.
Lipids 27, 266-269 (1992).

Except for the rare cases of monogenic hypercholesterolemia in humans and in rabbits, involving defects in low density lipoprotein (LDL) receptor function (1,2), the biochemical mechanisms underlying the elevation of LDL and/or cholesterol remain largely unexplained. Studies during the last two decades have shown that apolipoproteins and specific enzymes play a central role in plasma lipid metabolism. Mutations in structural genes, therefore, could produce polymorphic proteins resulting in modified rates of enzymatic reactions and subsequently defective catabolism of lipoproteins.

Rapacz and coworkers (3-5) have identified extensive apolipoprotein-B (apoB) polymorphism in pigs including eight *apoB* alleles. One of these (*Lpb*^{5}) showed association with inherited hypercholesterolemia and atherosclerosis. Although the *Lpb*^{5} animals develop hypercholesterolemia even on a low fat diet, they exhibit normal LDL receptor function (4). Although the *Lpb*^{5} allele was highly correlated with the hypercholesterolemic phenotype, the variations in apoB and cholesterol levels observed in the offspring of the hypercholesterolemic parents suggest that the hypercholesterolemic phenotype is of a *polygenic nature.* Consequently, it is likely that factors other than ApoB polymorphism contribute to the hypercholesterolemia and its phenotypic variation. This paper reports on the first investigation of lecithin:cholesterol acyltransferase (LCAT), in normal (LC) and genetically hypercholesterolemic (HC) pigs. The data reported in this study show that the LCAT activity in the plasma of HC pigs is considerably lower than that found in their normal counterparts. Additional studies suggest that the lower enzyme activity may be due, at least in part, to the presence of an LCAT inhibitor in the plasma of the HC pigs.

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Abbreviations: *apoB* or *Lpb*, locus with allelic genes coding for apolipoprotein-B; DFP, diisopropylfluorophosphate; CE, cholesteryl ester; FC, free (unesterified) cholesterol; HC, hypercholesterolemic; HDL, high density lipoprotein(s); LC, normocholesterolemic; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; TC, total cholesterol; LpB or apoB, pig lipoproteins with apolipoprotein-B; *Lpb*^{x} = *Lpb*^{1}, *Lpb*^{2}, *Lpb*^{3}, *Lpb*^{4}, *Lpb*^{6} and *Lpb*^{7}, apoB alleles other than *Lpb*^{5} or *Lpb*^{8}.

MATERIALS AND METHODS

Chemicals and reagents. [1,2-^{3}H]Cholesterol (40.7 Ci/mmole) was from Amersham (Arlington Heights, IL). Crystalline bovine serum albumin (essentially fatty acid free) and cholesterol standard and Aquacide IA were from Calbiochem (San Diego, CA). Enzymatic kits for cholesterol assays were from Boehringer Mannheim (Indianapolis, IN). All other chemicals were obtained from Fisher Scientific Company (Pittsburgh, PA) and were of reagent grade or better.

The animals. The animals used in this study were selected from the experimental herd of the Immunogenetics Laboratory at the University of Wisconsin, Madison, where the majority of the immunogenetically defined apolipoprotein mutant genes, including eight apoB alleles, have been identified (4) and are being preserved through propagation of a relatively small group of pigs. A total of 41 pigs, 23 females and 18 males, ranging from 5-36 months of age, were used. Preliminary studies on ten pigs indicated significant differences between the LCAT activity of the normal and hypercholesterolemic pigs with different *Lpb* genotypes. This observation prompted the increase of the sample size to include all available animals with elevated LpB to a total of 28; eight *Lpb*$^{5/5}$ homozygotes, five *Lpb*$^{5/8}$ and fifteen *Lpb*$^{5/x}$ heterozygotes. The group of animals with normal LpB levels was increased by random selection to a total of 13 pigs all carrying the *Lpb*^{x} alleles (*Lpb*^{1}, *Lpb*^{2}, *Lpb*^{3}, *Lpb*^{4}, *Lpb*^{6} and *Lpb*^{7}).

Collection of samples. Blood was collected into a syringe containing 10% Na_2 EDTA, pH 7.2 (1 mL/60 mL of blood, 0.004 M final concentration) from the jugular vein of pigs that had been fasted overnight, and the sample was cooled immediately to less than 4°C. Plasma was obtained by centrifugation at 2000 × *g* for 30 min at 4°C and shipped on wet ice to the Fort Worth laboratory within 24 hr by courier.

Preparation of lipoprotein-deficient plasma fractions. Endogenous LCAT activity was inhibited by the addition of diisopropylfluorophosphate (DFP; 1 mM final concentration) to the plasma. The lipoproteins were then separated by ultracentrifugal flotation for 48 hr upon

adjusting the density to 1.225 g/mL (6). The lipoprotein-deficient infranatants were isolated, dialyzed against 0.01 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.01% EDTA, and adjusted to the volume of the original plasma sample.

Measurement of cholesterol. Total cholesterol was determined with the Abbott VP (Chicago, IL) biochromatic analyzer using the Abbott reagent kits. Unesterified cholesterol in whole plasma and total cholesterol in the lipoprotein-depleted plasma fractions were determined using an enzymatic assay with reagents supplied by Boehringer Mannheim.

Measurement of LCAT activity. Individual plasma samples were analyzed according to the method of Glomset and Wright (7) which was modified to employ human plasma high density lipoprotein (HDL) instead of heat-inactivated plasma as the substrate. HDL from human plasma was prepared (8) and labeled with [^{3}H]-cholesterol as described previously (9). Briefly, the isolated HDL was treated with diisopropylfluorophosphate (DFP) to suppress endogenous LCAT activity. A mixture of bovine serm albumin and [^{3}H]cholesterol was prepared and added to the HDL preparation to result in approximately 500,000 dpm/mL. Bovine serum albumin was added to the substrate to a final concentration of 2%. The labeled HDL substrate solution was stored at 4°C and was stable for at least two months. This was ascertained by standard plasma samples being run along with the test samples for each run (10). The volume of the substrate used was 200 μL and the enzyme reaction was initiated by the addition of 5 μL of plasma. This high dilution of the plasma sample in the large substrate aliquot (1:40) was used in order to minimize the contribution of the free cholesterol introduced into the assay mix with the aliquots of the plasma. The incubation time for the LCAT assay was selected to yield a linear rate of cholesterol esterification so that no more than 10% of the total available free cholesterol was converted to cholesteryl ester. These conditions have been found to be adequate to assure the linearity and the reproducibility of the assay. All LCAT assays were performed using a single preparation of the [1,2-^{3}H]cholesterol labeled HDL substrate. The incubations (16 hr) were carried out in duplicates at 37°C with an average error of less than 5% between duplicates. The rate of cholesterol esterification (LCAT units) was calculated by multiplying the fractional rate (% cholesterol esterification/time) by the free cholesterol concentration present in the respective assay mixtures; including free cholesterol in the substrate (271 nmole of FC/mL) plus the free cholesterol originating from the aliquot of pig plasma (used as the enzyme source). Blank incubations (HDL substrate only) resulted in % cholesteryl ester values of 0.2%. These blank values were routinely subtracted from the assay results.

RESULTS AND DISCUSSION

Plasma lipids and LCAT assays. Plasma samples from a total of 41 pigs were used in this study. Of these, 13 were arbitrarily designated as normocholesterolemic (total cholesterol (TC) < 85 mg/dL) and 28 as hypercholesterolemic (TC > 98 mg/dL). The normocholesterolemic (LC) group was made up of animals with genotypes which carried the following apoB alleles: Lpb^1, Lpb^2, Lpb^3, Lpb^4, Lpb^6 and Lpb^7. The HC group consisted of $Lpb^{5/8}$ and $Lpb^{5/x}$ heterozygous and $Lpb^{5/5}$ homozygous animals. Total cholesterol (TC), free (unesterified) cholesterol (FC) and LCAT activity were determined. Table 1 shows the plasma cholesterol values and the LCAT activity for the normocholesterolemic (LC) and hypercholesterolemic (HC) pigs separated into male and female groups. Among the LC animals, TC ranged from 50 to 84 mg/dL (mean = 66 ± 9.2 mg/dL) and LCAT activity from 70 to 253 units (mean = 175 ± 45 units). In the hypercholesterolemic group, TC ranged from 98-409 mg/dL (mean = 249 ± 93 mg/dL) and LCAT activity from 38-195 units (mean = 79 ± 43 units). Figure 1 shows the LCAT activity (measured using an exogenous, substrate) as the function of TC in the plasma of LC pigs. A positive trend in LCAT activity was observed with increasing plasma TC as indicated by the linear correlation coefficient ($r = +0.54$; $p < 0.05$). However, the same relationship for the data obtained for the samples of the HC pigs revealed a *negative* trend ($r = -0.73$, $p < 0.001$) as shown in Figure 2.

The effectiveness of LCAT in the plasma has been assessed by either measuring its ability to esterify

TABLE 1

Plasma Cholesterol and LCAT Activity

Experimental group (no. in group)	Age (mo)	TC (mg/dL)	FC (mg/dL)	% EC	LCAT (LCAT units)
a) Normocholesterolemic pigs ($Lpb^{x/x}$)					
Males (7)	13.6 ± 6.3 (6-24)	60.3 ± 6.6 (50-68)	14.4 ± 1.9 (13-18)	75.5 ± 5.6 (64-80)	162 ± 51.8 (70-212)
Females (6)	11.5 ± 7.3 (6-24)	72.8 ± 7.1 (64-84)	18.8 ± 2.2 (16-22)	74.3 ± 3.32 (70-79)	188.8 ± 40.7 (137-253)
b) Hypercholesterolemic pigs ($Lpb^{5/x}$ and $Lpb^{5/5}$)					
Males (11)	12.5 ± 7.3 (54-27)	234.9 ± 80.8[a] (128-384)	67.3 ± 26.9[a] (30-124)	72.1 ± 3.1 (68-77)	65.5 ± 17.6[a] (42-102)
Females (19)	10.1 ± 7.7 (5-36)	257.8 ± 101[a] (98-409)	75.9 ± 36.2[b] (24-134)	71.6 ± 3.5 (64-77)	88.1 ± 52.1[a] (38-195)

[a]$p < 0.0005$; [b]$p < 0.005$; denotes significance of difference from values in the corresponding control group.

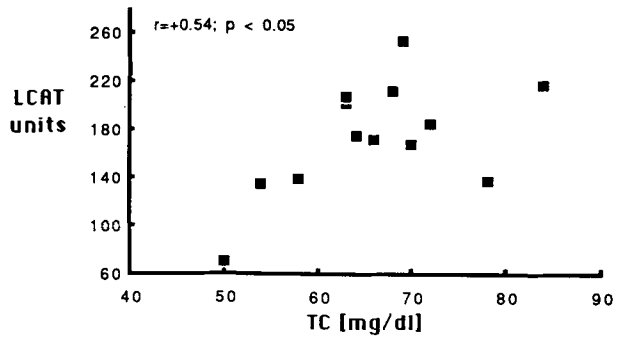

FIG. 1. LCAT activity as a function of total cholesterol (TC) concentration in the plasma of normocholesterolemic (LC) pigs. The data are representative of two separate sets of determinations.

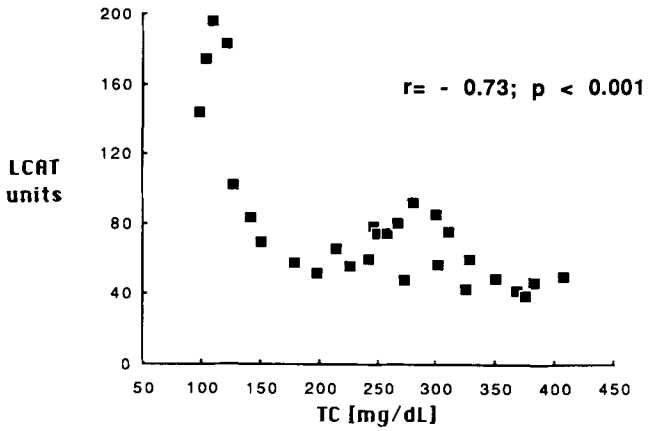

FIG. 2. LCAT activity as a function of total cholesterol (TC) concentration in the plasma of hypercholesterolemic (HC) pigs. The data are representative of two separate sets of determinations.

endogenous plasma cholesterol (10) or by the esterification of *exogenous* cholesterol in artificially prepared substrates (11). Although lower (12-14) and higher (14) rates of *endogenous* cholesterol esterification have been reported for a number of pathological states, the levels of circulating LCAT activity for these cases (as measured with an exogenous substrate) have been generally found to be normal. The few exceptions to these are congenital LCAT deficiency (15), parenchymal liver disease (16), and perhaps advanced renal disease (17). Therefore, it was unexpected to find significantly lower LCAT activity ($p < 0.0005$) in the plasma of hypercholesterolemic pigs (79 ± 43 units) as compared to their normocholesterolemic controls (175 ± 45 units). Examination of the LCAT activity (with an exogenous substrate), as a function of TC levels, revealed even more striking differences between the HC and LC pigs (Figs. 1 and 2). We have shown earlier that the circulating LCAT activity *increased* essentially linearly as a function FC (or TC) in a number of mammalian species (18). We have now confirmed such a trend in a group of 12 of 13 normocholesterolemic (LC) pigs (Fig. 1). Lower plasma cholesterol levels observed in males older than 12 months compared to females of the same age is in agreement with the general trend observed in both LC and HC pigs (3). However, the LCAT activity in the plasma of hypercholesterolemic (HC) pigs showed a *decreasing* trend with increasing TC levels especially up to TC values of 200 mg/dL (Fig. 2). Such a lower LCAT activity could be due to a number of factors including decreased synthesis or increased removal of the enzyme by the liver or to the presence of an LCAT inhibitor in the HC pigs. Only the latter possibility was investigated, as currently we have no means to measure LCAT enzyme protein levels directly by an immunoassay in pig plasma.

The lower LCAT levels particularly in the plasma of the HC pigs resulted in a lower percentage of esterified cholesterol in the plasma. Although the differences shown for % cholesteryl ester (CE) in Table 1 are not significant, the difference between HC and LC animals does become significant when the males and females are combined into one group (% CE = 75 ± 4.5 for the LC group and 71.8 ± 3.3 for the HC group; $p < 0.05$). Furthermore, there was a rather strong positive correlation ($r = 0.52$; $p < 0.005$) between LCAT activity and % CE in the plasma (Fig. 3).

Incubation studies. Preliminary experiments were conducted to investigate whether the lower LCAT activity in HC pigs was due to the presence of a circulating inhibitor. Plasma samples (1 mL each) from 10 HC pigs were pooled, treated with DFP and subjected to ultracentrifugation to obtain the lipoprotein-deficient infranatant ($d > 1.225$ g/mL). Increasing amounts of the dialyzed lipoprotein-deficient infranatants (obtained from HC and LC pigs) were preincubated (30 min; 25°C) with 100 μL of LC plasma containing active LCAT. Following the preincubation period, 5 μL aliquots were transferred to the assay mix to measure the residual LCAT activity. Although the lipoprotein-deficient infranatants contained a limited amount of total cholesterol (<10 mg/dL), this contributed less than 2% to the free cholesterol pool in the assay mix. The data presented in Figure 4 show that whereas the lipoprotein-deficient infranatant from the HC pigs inhibited LCAT activity, similar preparations obtained from the plasma of LC pigs had no such effect.

The etiology of hypercholesterolemia in the *Lpb*^{5} pigs has not yet been fully elucidated. Consequently, the present studies examined relevant aspects of lipoprotein metabolism to learn more about the mechanism of the

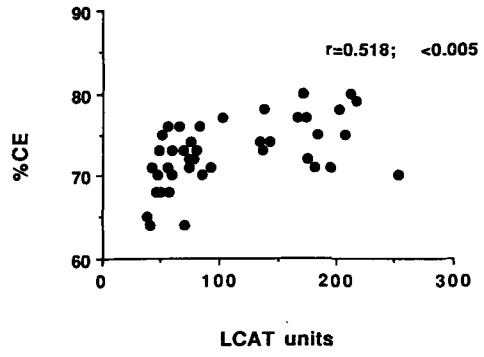

FIG. 3. Percent plasma cholesteryl esters as a function of CAT activity. Includes observations made with both normocholesterolemic (LC) and hypercholesterolemic (HC) pig plasma samples.

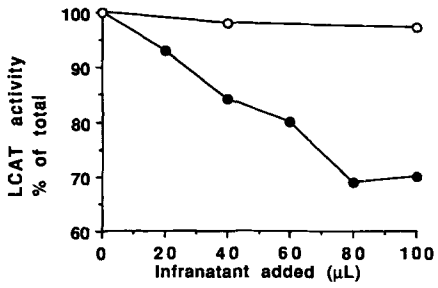

FIG. 4. Inhibition of LCAT activity in the plasma of a normocholesterolemic (LC) pig by lipoprotein-free infranatant prepared from the plasma of hypercholesterolemic (HC) pigs. Plasma samples pooled from HC pigs were treated with DFP and the lipoprotein-deficient infranatant (d > 1.225) was isolated by ultracentrifugation. Exhaustive dialysis followed (0.01 M Tris-HCl; pH 7.4 containing 0.15 M NaCl and 0.1% EDTA) to remove the unreacted DFP. Control infranatants from LC pigs were also prepared using the same DFP treatment and dialysis as for the HC infranatants and brought to the original volume of the plasma. Increasing amounts of the infranatants from HC pigs (■) and from LC pigs (□) were preincubated with 100 μL of LC plasma (30 min, 25°C). Following the incubation with the infranatants, 5 μL aliquots were transferred to the assay mixture to measure the residual LCAT activity. The data are representative of four separate experiments.

abnormal plasma cholesterol accumulation in these animals. The enzyme LCAT has been known to contribute to reverse cholesterol transport by converting the free cholesterol on the surface of the circulating lipoprotein particles to cholesteryl esters. As a result, most of the plasma cholesterol is made unavailable for exchange with membrane cholesterol pools; instead, it is targeted for removal by hepatic lipoprotein receptors. The physiological and clinical consequences of prolonged absence (15) or lower than normal (19,20) LCAT activity have been well documented and are characterized by the storage of free cholesterol in a number of tissues including the arteries (21). In this communication, we report for the first time, reduced LCAT activity associated with hypercholesterolemia and premature atherosclerosis in genetically inbred pigs. The mechanism underlying this decreased LCAT activity and its relationship to hypercholesterolemia and atherosclerosis is yet to be established.

ACKNOWLEDGMENTS

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Oxysterols in Cultured Bovine Aortic Smooth Muscle Cells and in the Monocyte-Like Cell Line U937

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Oxysterols in cultured bovine aortic smooth muscle cells and in the monocyte-like cell line U937 were analyzed by gas-liquid chromatography. The following products were detected: 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-α-epoxide, cholesterol-β-epoxide, cholestanetriol, and 20- and 25-hydroxycholesterol. The proportions of oxidized cholesterol varied from 1 to 3% of total cholesterol. The concentrations of oxysterols were lower when the smooth muscle cells were maintained in a vitamin E-enriched medium, and were higher in U937 cells when the cells were activated with phorbol myristate acetate. The cell oxysterol content appears to be regulated by factors that inhibit or enhance free radical generation. The concentrations of oxysterols found in cells may serve as an indicator of the extent of lipid peroxidation.
Lipids 27, 270–274 (1992).

The presence of oxygen radicals has been linked to a number of human diseases, and the damaging effects of free radicals, particularly in the vascular system, have attracted increasing attention (1,2). One of the major consequences of free radical mediated reactions is lipid peroxidation. Unsaturated fatty acids, as constituents of membrane phospholipids, are easy targets of oxidative processes and several methods have been developed to measure fatty acid oxidative end products (1). Cholesterol, a major cell membrane component of animal tissue, also can easily undergo oxidation, but measurement of oxidized cholesterol is rarely performed. Recent studies have shown that cholesterol can be oxidized after photoxidation of red blood cell membranes (3,4) or upon CCl_4 poisoning of rat liver (5). Using our recenty developed method which allows simple and reliable quantification of oxysterols and cholesterol by gas-liquid chromatography (GLC) (6), we analyzed the oxysterol contents of cultured cells derived from the vascular system, namely bovine aortic smooth muscle cells and U937 cells, a monocyte-like cell line that grows easily and generates oxidant species when activated. Indeed, it seemed particularly interesting to investigate oxysterol formation in these cells because oxysterols can modify various parameters of vascular physiology. They recently have been shown to alter endothelial permeability (7), and to change arachidonic acid metabolism and fatty acid distribution in smooth muscle cells (8,9). The present report describes the oxysterol contents of cultured bovine aortic smooth muscle cells and U937 cells, and the effects of protective culture conditions and oxidant stress on the formation of oxidized cholesterol.

MATERIALS AND METHODS

Reagents. Cholesterol, cholestanol, cholestanetriol, cholesterol-α-epoxide, 20-hydroxycholesterol, 7-ketocholesterol and 19-hydroxycholesterol, butylated hydroxytoluene (BHT), phorbol myristate acetate (PMA) and α-tocopherol were obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol-β-epoxide came from Research Plus (Bayonne, NJ), and 25-hydroxycholesterol and 7α- and 7β-hydroxycholesterol were purchased from Steraloids Inc. (Wilton, NH). All standards were found to be pure as determined by GLC analysis. Culture reagents were obtained from Flow Laboratories (Irvine, U.K.).

Cell cultures. Bovine aortic smooth muscle cells were cultured as described elsewhere (8). Cells from explants of bovine aortic media were used up to the eighth passage. The culture medium used was RPMI 1640 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) supplemented with 10% heat-inactivated fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 1 μg/mL amphotericin B and 2 mM L-glutamine. The same batch of fetal bovine serum was used throughout the study. The monocyte-like cell line U937 was obtained from the European cell culture collection (Salisbury, U.K.). Cell suspensions were maintained at a density ranging from 0.3 to 0.9×10^6 cells/mL in the same culture medium used for smooth muscle cells.

Cultures in vitamin E-enriched medium. Smooth muscle cells were obtained as described above. After the first passage, the cultures were divided into two parts. The control cells were maintained in the usual culture medium, and the other cells were maintained in the culture medium containing 1 μg/mL of α-tocopherol. Vitamin E stock solutions at 0.1 mg/mL of acetone were stored at −20°C and diluted in pure ethanol. The vitamin solution was added in the medium each time the medium was changed, throughout the maintenance of the culture. The final ethanol concentration was not higher than 0.5% (v/v) and did not affect cell viability.

Activation of U937 cells with PMA. The cells were rinsed once with RPMI without fetal bovine serum by centrifugation, and resuspended in the same medium. The PMA was stored at −20°C in 1 mg/mL of acetone, diluted in ethanol at the desired concentrations, and added into the medium. Cell suspensions were divided into 75-cm^2 culture flasks and incubated for 60 min at 37°C. At the end of the incubation, the cell suspensions were recovered and the adhering cells were detached with 10 mL per flask of a trypsin-EDTA solution.

Lipid extraction. Total lipids were extracted from the samples by the Folch procedure (10). Smooth muscle cells

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Abbreviations: Abbreviations: BHT, butylated hydroxytoluene; GLC, gas-liquid chromatography; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid); PMA, phorbol 12-myristate 13-acetate; TLC, thin-layer chromatography.

Nomenclature: Cholestanetriol, cholestane-3β,5α,6β-triol; cholestanol, 5α-cholestan-3β-ol; cholesterol, cholest-5-en-3β-ol; cholesterol-α-epoxide, 5,6α-epoxy-5α-cholestan-3β-ol; cholesterol-β-epoxide, 5,6β-epoxy-5α-cholestan-3β-ol; 7α-hydroxycholesterol, cholest-5-ene-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-ene-3β,7β-diol; 19-hydroxycholesterol, cholest-5-ene-3β,19-diol; 20-hydroxycholesterol, cholest-5-ene-3β,20α-diol; 25-hydroxycholesterol, cholest-5-ene-3β,25-diol; 26-hydroxycholesterol, cholest-5-ene-3β,26-diol; 7-ketocholesterol, 3β-hydroxycholest-5-en-7-one.

from 5-75 cm^2 flasks were trypsinized and pooled, and 10-mL samples of the cell suspension were extracted with 30 mL of chloroform/methanol (2:1, v/v) containing 0.002% BHT (w/v) and the internal standards for the quantification of the cholesterol oxides (10 μg of 19-hydroxycholesterol) and cholesterol (200 μg of cholestanol). U937 cell suspension was similarly extracted—30×10^6 cells were necessary for each sample. Approximately 1 g of aortic media fragments was extracted with 20 mL of chloroform/methanol (2:1, v/v) containing BHT and the internal standards. The suspension was mixed vigorously for 30 min, and once the organic phase was recovered, the extraction was repeated under the same conditions. Lipids extracts were evaporated under nitrogen.

Quantification of cholesterol and oxysterols. Lipids extracts were saponified overnight at room temperature with 20 mL of 1N KOH in methanol. The unsaponifiable fractions were deposited on a silica thin-layer chromatography (TLC) plate and developed in hexane/diethyl ether (70:30, v/v) to separate oxysterols from cholesterol. The extracts of scraped silica gel were derivatized as previously described (6). The sterols were quantified by GLC analysis on a Girdel 30 gas chromatograph (Suresnes, France) with a flame ionization detector. The GLC conditions were: A 30-m fused silica DB5 column (J&W Scientific, Folsom, CA) with a film thickness of 1.0 μm; oven temperature, 280°C and 270°C for oxysterols and cholesterol, respectively; detector at 310°C; injector at 300°C; flow rate of helium, 1 mL/min; and pressure 12 psi.

Verification of the identity of the substances detected by GLC analysis. To verify the identity of the substances detected by GLC by relative retention time, the following experiment was performed. Lipid extracts from 10 pooled 75-cm^2 culture flasks were processed as described above up to the TLC step. Once the silica was recovered and extracted, the extracts were deposited again on a silica TLC plate, and developed in 100 mL of hexane/diethyl ether/ethyl acetate (50:50:50, v/v/v). Once developed and dried, the plates were sprayed with rhodamin (1 mg/mL in ethanol). Four bands of silica were recovered and extracted separately. These bands corresponded to migration areas of standards run in parallel. The extracts were derivatized and analyzed under usual GLC conditions.

Statistics. Results are expressed as mean ± SD. The significance of differences between groups was assessed by using one-way analysis of variance and Student's *t*-test.

RESULTS AND DISCUSSION

We quantified cholesterol and oxysterols with good reproducibility and satisfactory recoveries using our method (6). Different oxysterols were detected in the lipid extracts obtained from smooth muscle cells and U937 cells; Table 1 links the range of amounts found in the samples and the proportions of the different oxysterols expressed as percentages of the total cholesterol contained in the sample. Under our conditions, the detection limit was 0.05 μg/18×10^6 U937 cells or 0.05 μg/mg protein of smooth muscle cells. Typical GLC profiles are shown in Figure 1. As shown in Table 1 and Figure 1, eight compounds were detected in the smooth muscle cell cultures. The most abundant species were cholesterol-β-epoxide, 7-ketocholesterol and cholesterol-α-epoxide. The high standard deviations were due to differences in the cultures analyzed. In spite of these large variations, we consistently found low amounts of 7α- and 7β-hydroxycholesterol in these cells. In contrast, both substances were more abundant in U937 cell extracts, where 7-ketocholesterol was consistently found in the highest proportion. In this cell line, 25-hydroxycholesterol was not detected. As shown in Table 1, the profile of oxysterols detected in the cells differed from the profile of these compounds in the serum. In light of the differences noticed between the two cell types, and because both cultures were maintained in the same medium, it seems probable that the oxysterols found in the cells do not only originate from the medium.

Low amounts of oxysterols in the samples and lack of sensitivity prevented us from using mass spectrometry to verify the identity of the substances detected by GLC (6). Therefore, we analyzed the samples using a further TLC step. Figure 2 shows the migration of pure compounds in the system used. For the smooth muscle cell extracts, silica gel was scraped in four bands as shown in Figure 2 and the content of each band was analyzed by GLC. Figure 2 shows a typical GLC profile obtained for band III from a cell sample. This band corresponded

TABLE 1

Quantification of Oxysterols in Cultures of Smooth Muscle Cells and U937 Cells[a]

	Smooth muscle cells	U937 cells	Fetal calf serum
Cholesterol	0.23 ± 0.06	0.14 ± 0.03	0.31 ± 0.01
7α-Hydroxycholesterol	0.15 ± 0.07 (0.06)	0.27 ± 0.10 (0.19)	0.40 ± 0.03 (0.13)
7β-Hydroxycholesterol	0.12 ± 0.06 (0.05)	0.27 ± 0.10 (0.19)	0.40 ± 0.01 (0.13)
7-Ketocholesterol	1.49 ± 0.51 (0.64)	1.08 ± 0.41 (0.76)	1.78 ± 0.04 (0.57)
Cholesterol-α-epoxide	1.18 ± 0.49 (0.50)	0.34 ± 0.12 (0.24)	0.30 ± 0.02 (0.09)
Cholesterol-β-epoxide	1.89 ± 0.82 (0.81)	0.51 ± 0.09 (0.36)	0.08 ± 0.01 (0.02)
Cholestanetriol	0.25 ± 0.12 (0.11)	0.17 ± 0.09 (0.12)	N.D.
20-Hydroxycholesterol	0.66 ± 0.29 (0.28)	0.53 ± 0.13 (0.37)	0.07 ± 0.01 (0.02)
25-Hydroxycholesterol	0.48 ± 0.17 (0.20)	N.D.	0.06 ± 0.01 (0.02)

[a]Results are expressed in g/sample of cells or g/mL of serum for cholesterol, and in μg/sample of cells or μg/mL of serum for oxysterols. Numbers in parentheses indicate the proportion for each oxysterol expressed as percentage relative to total cholesterol. Each sample consisted of 5-75-cm^2 culture flasks for smooth muscle cells and of 30×10^6 cells for U937 cells. N.D., not detectable. Mean ± SD (n = 15 for the cell extracts, n = 3 for the serum).

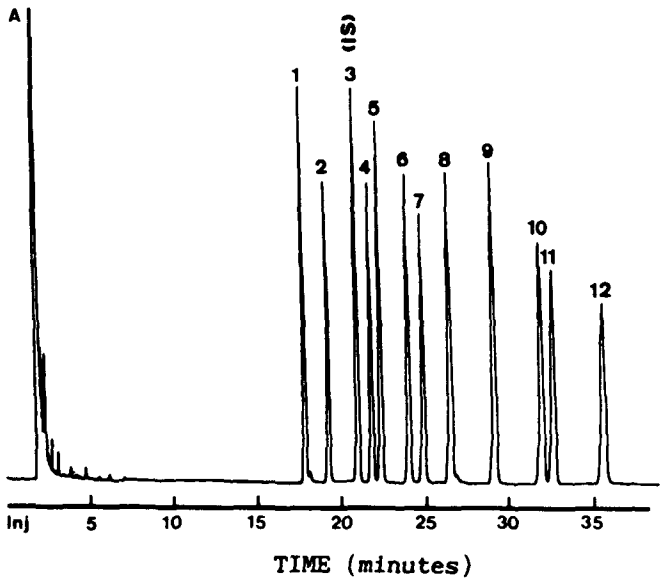

FIG. 1A. GLC profiles of standards.

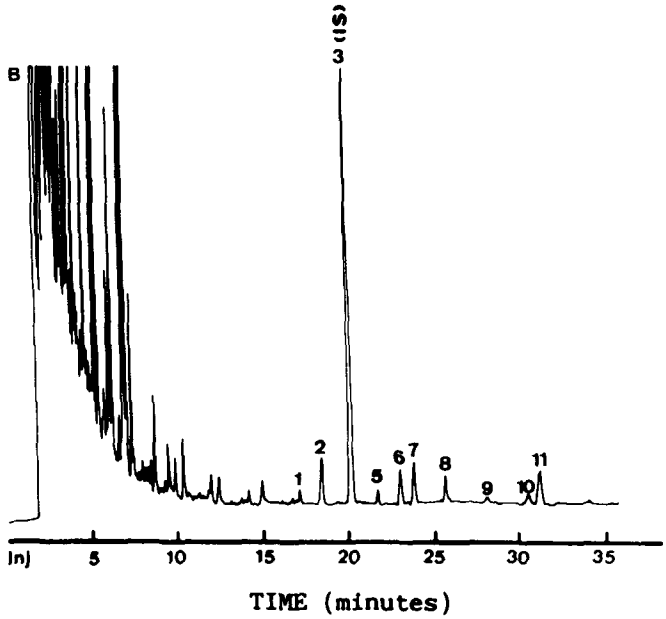

FIG. 1B. Smooth muscle cells.

to the area of migration of 7-ketocholesterol, cholesterol-α-epoxide, cholesterol-β-epoxide and 25-hydroxycholesterol. The GLC profile shows four peaks with the same retention times as the four oxysterols. Thus the presence of these substances in the cell samples was confirmed. Using this method we could obtain confirmation of the presence of the eight oxysterols detected by GLC.

We also performed experiments to verify that the oxysterols were not produced artifactually during analysis.

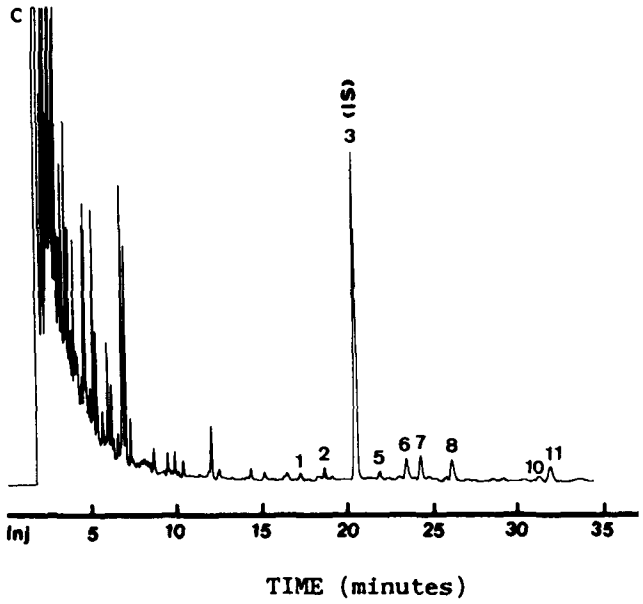

FIG. 1C. U937 cells. 1, 7α-Hydroxycholesterol; 2, cholesterol; 3, 19-hydroxycholesterol; 4, cholesta-3, 5-diene-7-one; 5, 7β-hydroxycholesterol; 6, cholesterol-β-epoxide; 7, cholesterol-α-epoxide; 8, 20-hydroxycholesterol; 9, cholestantriol; 10, 25-hydroxycholesterol; 11, 7-ketocholesterol; 12, 26-hydroxycholesterol.

The results are presented in Table 2. The cholesterol added to the sample was first purified by TLC to remove any contaminating oxysterols. As seen in the table, when a cell sample was spiked with cholesterol no increase in the amounts of oxysterols could be observed. Cholesterol was stable throughout the analysis, and the oxysterols detected were not derived by spontaneous oxidation occurring during sample processing.

Since the sum of oxysterols represented a significant portion of cell sterols, we analyzed the oxysterol content of media from bovine aortas and compared them to the data obtained on cells maintained *in vitro*. The results for three different aortas are shown in Table 3. Only four oxysterols, in relatively low amounts, could be detected in each sample, since the sum of these compounds represented only 0.4% of total sterol. There were fewer primary oxysterols detected than in cells maintained *in vitro*, since 7-ketocholesterol was not always present and there was no 7α- and 7β-hydroxycholesterol. Thus, as expected, in cultured cells, cholesterol is more oxidized. Indeed, cell cultures are exposed to intense oxidative conditions, such as high O_2 concentrations. Furthermore, culture media contain iron and copper salts or HEPES buffer that can enhance oxygen radical generation (11,12).

The oxysterol content of cultured cells was investigated next to see whether it can be modulated by factors that either inhibit or stimulate free radical formation. Smooth muscle cells were cultured in vitamin E-enriched medium. The concentration chosen (1 μg/mL) did not affect cell growth, while at 2 μg/mL, vitamin E blocked cell division without any sign of cytotoxicity. The cell content of oxidized cholesterol was significantly reduced in cells maintained in vitamin E-enriched medium. After 13 days of repeated exposure to α-tocopherol, the ratio of oxysterols to cholesterol dropped from 3.5% (controls) to 1.7%. Reductions in the total amount of oxysterols varied from 50 to

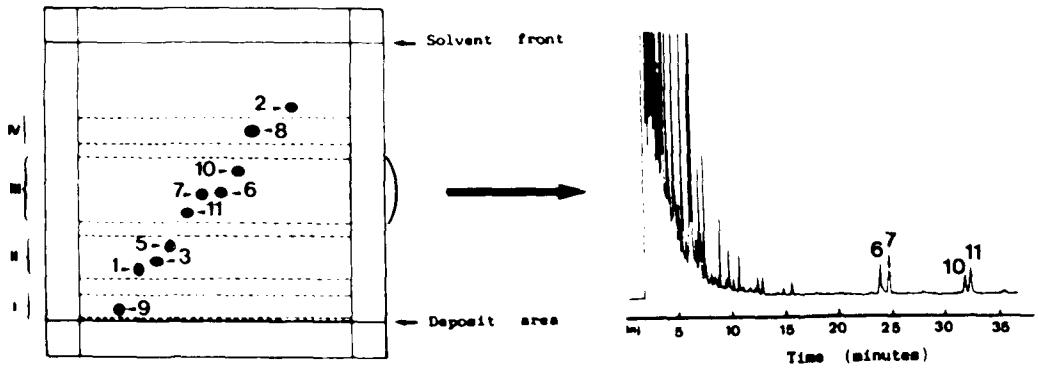

FIG. 2. Verification of the identity of the substances detected by GLC analysis. Unsaponifiable fractions of cell extracts were developed in hexane/diethyl ether/ethyl acetate (50:50:50, v/v/v) using silica gel plates, and standards were simultaneously run on a separate plate (left). For the cell samples, four bands of silica corresponding to migration areas of standards were recovered, extracted, and analyzed by GLC. A typical profile of the content of band III of a cell sample is shown (right). Oxysterols have been numbered in the same way as in Figure 1.

TABLE 2

Control of the Stability of Cholesterol During the Analysis[a]

	Cell extract	Cell extract + cholesterol
Cholesterol	0.18 ± 0.05	1.54 ± 0.62
7α-Hydroxycholesterol	0.11 ± 0.02	0.10 ± 0.01
7β-Hydroxycholesterol	0.07 ± 0.01	0.06 ± 0.02
7-Ketocholesterol	1.39 ± 0.25	1.46 ± 0.25
Cholesterol-α-epoxide	1.30 ± 0.52	1.28 ± 0.45
Cholesterol-β-epoxide	1.63 ± 0.53	1.68 ± 0.60
Cholestanetriol	0.32 ± 0.18	0.24 ± 0.20
20-Hydroxycholesterol	0.34 ± 0.08	0.32 ± 0.10
25-Hydroxycholesterol	0.58 ± 0.10	0.61 ± 0.12

[a]Smooth muscle cells from 10–75-cm^2 flasks were pooled and lipids were extracted. The sample was divided into two parts and pure cholesterol was added into one of the samples. Analysis was performed as described in Materials and Methods. Results are expressed in g/sample for cholesterol and μg/sample for oxysterols. Mean of four experiments ± SD.

85%. The oxysterols were not equally reduced, as shown in Figure 3. Oxysterols oxidized on the side chain were not reduced as much as the others. The reductions noted for 20- and 25-hydroxycholesterol varied from 0 to 33%, while they were consistently higher than 50% for the other products.

When U937 cells were exposed for a short time to 100 ng/mL of PMA, the oxysterol content of the cells increased markedly, as shown in Figure 4. The concentration of 7α- and 7β-hydroxycholesterol doubled after exposure to PMA, and an increase in 20-hydroxycholesterol, 7-ketocholesterol and the two cholesterol epoxide isomers was also observed. When BHT was added to the incubation medium containing PMA, the PMA induced increases were suppressed, as shown in Figure 4. Thus, the increases noted for different oxysterols after PMA stimulation seem to be the result of free radical generation.

Our experiments have shown that cell oxysterols derive partly from radical generation and spontaneous cholesterol autooxidation. Nevertheless, it is probable that they have diverse origins and that they also partly derive from enzymatic pathways. For instance, the failure to reduce 20- and 25-hydroxycholesterol after repeated exposure to vitamin E suggests that they do not derive from radical generation. Our method includes saponification of the lipid extract, and the data obtained do not provide any information on the distribution between free and esterified sterols. Also, we only analyzed the total amount of oxysterols and cholesterol present in the cells and the data do not provide any insight into the distribution of the sterols between membranes and cytosols. It would be of interest to focus more precisely on the oxysterol content of plasma membranes and to assess the modifications

TABLE 3

Quantification of Cholesterol and Oxysterols in Media from Bovine Aortas[a]

	Cholesterol	Cholesterol-β-epoxide	Cholesterol-α-epoxide	20-Hydroxycholesterol	7-Ketocholesterol
Aorta 1	0.65 ± 0.01	1.19 ± 0.07	1.26 ± 0.08	0.23 ± 0.03	0.17 ± 0.01
Aorta 2	0.72 ± 0.01	1.08 ± 0.06	0.70 ± 0.05	0.18 ± 0.03	0.28 ± 0.02
Aorta 3	0.71 ± 0.01	1.15 ± 0.06	1.08 ± 0.06	0.16 ± 0.02	N.D.

[a]Results are expressed in g/g of tissue for cholesterol and in μg/g of tissue for oxysterols. Mean ± SD (n = 3); N.D., not detected.

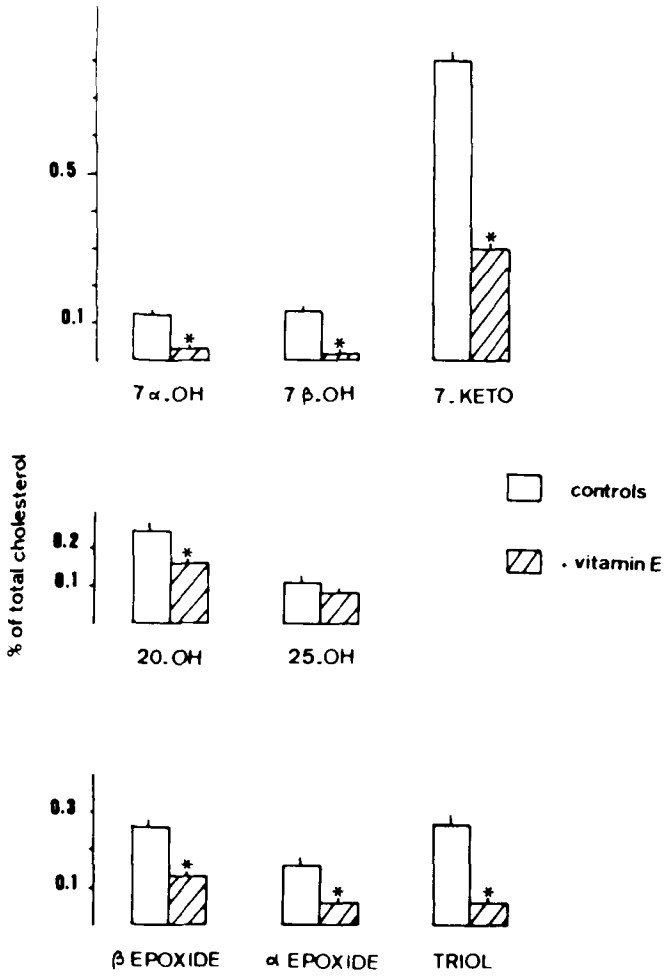

FIG. 3. Effect of vitamin E enrichment of culture medium on the oxysterol content of smooth muscle cells. Vitamin E was added at 1 μg/mL to the culture medium each time the medium was changed throughout the maintenance of the culture. Each oxysterol is expressed as a percentage relative to total cholesterol. The data are the result of a typical experiment. Similar results were found in three separate experiments. *Significantly different from controls at $P < 0.05$. Abbreviations: 7α-OH, 7α-hydroxycholesterol, 7β-OH, 7β-hydroxycholesterol; 7-keto, 7-ketocholesterol; 20-OH, 20-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; β-epoxide, cholesterol-β-epoxide; α-epoxide, cholesterol-α-epoxide; triol, cholestanetriol.

of plasma membrane cholesterol after exposure to oxidant or protective factors. Indeed, it has been shown that oxysterol inclusion in the plasma membranes can modify membrane fluidity (13), permeability to ions (14,15) and enzymatic activities (16). Our results show that changes were detectable under both conditions, either protective or oxidant. Since we analyzed whole cell extracts, an underestimation of the plasma membrane changes is probable. Nevertheless, it seems that the quantification of oxysterols can serve as an important indicator of lipid peroxidation and that our method is a reliable tool in assessing the changes induced by free radical formation.

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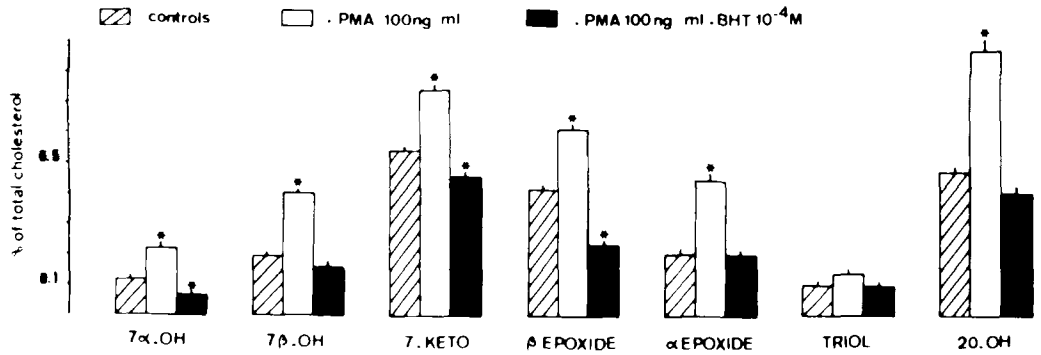

FIG. 4. Effect of PMA induced activation on oxysterol content of U937 cells. U937 cells were incubated at 37°C for 60 min in RPMI without fetal bovine serum either alone (controls) or with PMA or PMA plus BHT at the concentrations indicated above. The oxysterols are expressed as percentage of total cholesterol. The data are the results of a typical experiment. Similar data were obtained in three separate experiments. *Significantly different from control value at $P < 0.05$. The abbreviations used are the same as in Figure 3.

Phospholipids in *Trypanosoma cruzi:* Phosphoinositide Composition and Turnover

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The polyphosphoinositides from *Trypanosoma cruzi* were isolated by preparative thin-layer chromatography (TLC) and identified. When myo-[^{3}H]inositol was present in the culture medium for five days, analyses showed the presence of phosphatidylinositol (PI), lysophosphatidylinositol (lysoPI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). Short-term incubation with $^{32}P_i$ led to higher percentages of incorporation into phosphatidylethanolamine (PE), lysophosphatidylethanolamine (lysoPE) and PI compared to the other glycerophospholipids. The phosphoinositides (PI, PIP and PIP_2) contained a larger proportion of unsaturated than saturated fatty acids. High proportions of 18:2 were found in the three phosphoinositides analyzed, whereas the major saturated fatty acid was 18:0. Water-soluble inositol phosphates (IP, IP_2 and IP_3) were also identified.
Lipids 27, 275–278 (1992).

Phosphoinositides are minor constituents of eukaryotic cells and are located in the plasma membrane. Phosphatidylinositol (PI) is the most abundant representative, whereas phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) often occur in trace amounts only (1,2). External signals such as hormones, neurotransmitters, growth factors, or light can cause changes in phosphatidylinositol metabolism in many organisms (1–5). In the parasitic protozoa, the role of phosphoinositides is not well understood. Some of the trypanosomatidae are pathogenic, and a most important representative is the etiological agent of American trypanosomiasis (Chagas' disease) with a life cycle involving mammalian and insect hosts. Both, the lipid composition of *Trypanosoma cruzi* and the presence of phosphatidylinositol (PI) in this organism have been reported previously (6). However, the presence of polyphosphoinositides had not been demonstrated. In our study on *Trypanosoma cruzi*, various forms of phosphoinositides were found, and the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) was examined.

MATERIALS AND METHODS

Organisms. The Tulahuen strain of *Trypanosoma cruzi* was used in this study. The epimastigote forms were grown at 28°C in a modified Warren medium (7) consisting of 37 g/L Brain/Heart Infusion (Merck, Darmstadt, Germany), 0.1% hemine (Sigma Chemical Co., St. Louis, MO) using 1 g of hemine in 50 mL of 0.2 M NaOH, 10% calf serum (GEN S.A., Buenos Aires, Argentina) and 1,000,000 U penicillin per 4×10^7 parasites or cells/mL. Growth was followed by measuring the weight of the harvested cells and the number of mobile cells per mL of culture medium. Cells in the logarithmic phase of growth were harvested by centrifugation at $6,400 \times g$ for 10 min and washed three times with Krebs-Ringer-TRIS (KRT) buffer (25 mM HCl-TRIS, pH 7.2; 1.2 mM $MgSO_4$; 2.6 mM $CaCl_2$; 4.8 mM KCl; 120 mM NaCl; and 100 mM glucose).

Radioisotope incorporation. Millipore sterilized myo-2-[^{3}H]inositol (1.2 μCi/mL) was added to the culture medium and growth was allowed to proceed for 5 days at 28°C. The parasites were then harvested according to the method described above, and the lipids were extracted. In other experiments, the cells harvested in the logarithmic phase were incubated with [^{32}P]orthophosphate (100 μCi/250 mg of cells) at 37°C for 3 hr. Cells were then harvested by centrifugation at $500 \times g$ for 20 min and processed for lipid extraction.

Lipid extraction. Total lipids were extracted from the washed parasites according to Bligh and Dyer (8). The lipid extract was washed with neutral upper-phase chloroform/methanol/0.1 M KCl (3:48:47, v/v/v) (9). After washing, the lipid extract was dried under a stream of nitrogen and redissolved in a suitable volume of chloroform/methanol (9:1, v/v).

Phospholipid separation and quantification. Phospholipids were separated by preparative thin-layer chromatography. Glass plates (20 × 20 cm) were coated with a slurry of 40 g Silica Gel H (Merck) in 90 mL of a solution of 0.01% Na_2CO_3 and 2 mM EDTA. Potassium oxalate, 1%, was added according to Jolles *et al.* (10). Prior to use, the plates were activated at 110°C for 60 min. An aliquot of the total lipid extract was applied onto the chromatoplate and developed with solvent A, chloroform/methanol/acetic acid/water (50:30:8:4, by vol), or solvent B, chloroform/methanol/4N NH_4OH (54:42:12, v/v/v). The developed chromatograms were exposed to iodine vapors, marked, and the fractions were removed from the plates for phosphorus determination (11,12).

The position of radiolabeled lipids was determined by autoradiography on Agfa-Gevaert Curix X-ray film. Spots were scraped off the plates, and fractions were counted in a liquid scintillation counter (6,13).

Gas-liquid chromatography (GLC) and thin-layer chromatography (TLC) of methyl esters of fatty acids. Fatty acid methyl esters were prepared from the lipid extract with 14% borontrifluoride in methanol (14). The methyl esters of fatty acids were separated by $AgNO_3$-TLC on the basis of their degree of unsaturation as described by Cook (15). The methyl esters were analyzed by GLC on a Varian 2100 gas chromatograph equipped with a flame-ionization detector (Varian, Palo Alto, CA). A column with 10% SP-2330 on 100/120 of Chromosorb WA W (Supelco, Bellefonte, PA) was operated at 180–190°C; the carrier gas was nitrogen. Peaks were identified by comparison with

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Abbreviations: DAG, diacylglycerol; EDTA, (ethylenedinitrilo) tetraacetic acid; GLC, gas-liquid chromatography; KRT buffer, Krebs-Ringer-Tris buffer: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PKC, protein kinase C; PS, phosphatidylserine; TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TLC, thin-layer chromatography.

those of standard fatty acid methyl esters. When standards were unavailable, peaks were assigned based on semilogarithmic plots of their relative retention times.

Elution of phosphatidylinositol from silica gel. Radioactive and nonradioactive phosphatidylinositol were eluted from thin-layer chromatograms according to the Holub procedure (16).

Incorporation of phosphorus into PI from [γ-^{32}P]ATP. *Trypanosoma cruzi* epimastigote forms homogenate was prepared from 200 mg cells in 10 vol (w/v) of 25 mM TRIS-HCl, pH 7.4, 0.30% Triton-X100 and centrifuged for 10 min at 800 × *g* (5°C) to remove heavy particulate matter. The supernatant was again centrifuged for 30 min at 12,000 × *g* (5°C). According to the method of Traynor-Kaplan *et al.* (17), 104 mg of the last sediment was resuspended in 25 mM TRIS-HCl, pH 7.4, to carry out the phosphorylation (18) and was then added to a reaction mixture containing 17 mM $MgCl_2$, 30 mM 2-mercaptoethanol, 0.15% Triton-X100 and 1 mM ATP[γ-^{32}P]ATP (specific activity 500–1000 cpm/pmol). Following incubation for 20 min at 30°C, the reaction was terminated by the addition of a cooled solution of nonradioactive 7 mM ATP and 20 mM (ethylenedinitrilo) tetraacetic acid (EDTA). Acidified methanol/chloroform was then added to extract the phospholipids.

Materials. All solvents (Merck) were of analytical grade. Phospholipid standards were obtained from Sigma Chemical Co.; [^{32}Pi]phosphoric acid, carrier-free, and [γ-^{32}P]ATP were kindly provided by Dr. Luis Beaugé, Instituto Martín Ferreyra (Córdoba, Argentina). Myo-[^{3}H]inositol (12.8 Ci/mmol) was purchased from New England Nuclear (NEN). Polyphosphoinositides were prepared from ox brain as described by Lees (19).

RESULTS AND DISCUSSION

[^{32}P]P_i was mainly incorporated into phosphatidylethanolamine (PE), lysoPE and PI. Table 1 illustrates the [^{32}P]P_i incorporation into lipids which were separated on oxalate impregnated silica gel thin-layer plates using chloroform/methanol/4N NH_4OH (54:42:12, v/v/v) as developing solvent. In addition significant quantities of the polyphosphoinositides PIP and PIP_2 and lysoPI were identified for the first time in *T. cruzi.*

The phosphoinositides accounted for 14.71% of total cellular lipid phosphorus (500–600 μgP_i/g cells) and were distributed as follows: PI, 63%; PIP, 10%; PIP_2, 8%, and lysoPI, 19%. (The phosphorus content in μgPi/g cells, in PIP and PIP_2 was divided by 2 and 3, respectively.) The PI content in respect to total phospholipids was similar to values found by others (6,20). Although phosphatidylinositol constitutes only 9.30 ± 1.61% of the total cellular phospholipids, and phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate are represented at even smaller percentages, [^{32}P]P_i was substantially incorporated into these lipids, and they showed a high turnover rate.

In previous studies, PC, PE and PI were the major phospholipids that were identified in whole *T. cruzi* epimastigotes (6,20,21) and in other flagelates (22,23). In our study, phosphatidylcholine (PC), PE, PI and lysoPE (10.46 ± 3.95%) were the major phospholipids found; the relative proportions of PC (21.00 ± 3.00%) and PE (11.06 ± 1.85%) were somewhat different from those reported earlier. Phosphatidic acid (PA) (5.00 ± 1.50%), as previously shown (20), lysoPC (4.43 ± 1.50%) and the polyphosphoinositides described earlier were also identified in *T. cruzi.*

TABLE 1

Phospholipid Turnover and Phosphoinositide Composition of *Trypanosoma cruzi* Epimastigote Forms[a]

Phospholipids	R_f	% of Incorporation [^{32}P][b]	% of Incorporation [^{3}H]inositol[c]
PE	0.75	30.0 ± 4.8	—
PC	0.62	8.5 ± 3.8	—
PI	0.55	13.9 ± 2.2	94.8 ± 3.0
LysoPE	0.52	27.4 ± 3.8	—
LysoPC	0.45	1.9 ± 1.0	—
LysoPI	0.39	4.3 ± 0.6	1.7 ± 1.3
PIP	0.33	8.0 ± 2.0	1.8 ± 0.4
PIP_2	0.14	6.0 ± 1.6	1.7 ± 0.7

[a]Results are expressed as mean ± SE for n = 3. Phospholipids from *T. cruzi* epimastigotes were resolved by TLC using chloroform/methanol/4N NH_4OH (54:42:12, v/v/v) for developing.

[b][^{32}P]P_i (100 μCi/250 mg of cells) was incubated with epimastigote forms (3.0 × 10^7/mL) for 3 hr at 37°C. Phospholipids and radioactivity were determined as described in Materials and Methods.

[c]Myo-[^{3}H]inositol 1.2 μCi/1.0 × 10^6 cells were incubated for 5 days.

An unidentified phospholipid fraction (5.00 ± 1.50%) that migrated between PIP_2 and the origin was present in whole cells. However, structural characterization of this unidentified phospholipid has not yet been completed.

Inositol phospholipids and phosphatidic acid are of interest because of their response to cell stimulation in various types of cells (24,25). Preliminary experiments with *T. cruzi* cells showed similar effects (Machado de Domenech, E.E., Garcia, M., Garrido, M., and Racagni, G., manuscript in preparation). Furthermore, the presence of PIP and PIP_2 would indicate the presence of kinases responsible for PI phosphorylation in *T. cruzi,* which had not been demonstrated previously (see below).

^{3}H-Labeled lipid bands were observed migrating with PI, PIP and PIP_2 in cells grown for five days in the presence of myo-[^{3}H]inositol (not shown). These bands seem to represent authentic phosphoinositides. Our long-term (five days) myo-[^{3}H]inositol labeling experiments (Table 1) indicate that there is a very small amount of inositol incorporated into PIP and PIP_2 compared to the large PI pool in epimastigotes of *T. cruzi,* as has been observed in other membranes (22,26). One to two percent of the myo-[^{3}H]inositol added was incorporated into epimastigotes. The PIP_2 amount was approximately equal to PIP in this protozoa; however, in *C. fasciculata* and yeast, PIP was much more abundant than PIP_2 (22).

In other experiments, radioactive phosphate from [γ-^{32}P]ATP was incorporated into PI to form a phosphorylated product that co-migrated with PIP in thin-layer chromatography. PIP on the plate was localized by autoradiography and by comparison with an authentic standard (results not shown here).

Labeling of PI, PE and lysoPE may indicate that these lipids were formed from rapidly labeled precursors, as suggested by Palmer (22) for *C. fasciculata.* Another possibility is that phosphatidylethanolamine serves as the precursor of phosphatidylcholine *via* methylation as has been

TABLE 2

Fatty Acid Composition of Total Lipids and Phosphoinositide Fractions from *Trypanosoma cruzi* (epimastigote forms)

Fatty acid	% Total fatty acid methyl esters			
	Total Lipids[a]	PIP_2[b]	PIP[c]	PI[d]
14:0	1.70 ± 0.32	2.8	1.6	0.7
15:0	1.12 ± 1.14	2.9	2.2	0.4
16:0	16.12 ± 2.12	9.3	13.8	12.1
16:1	6.20 ± 2.25	2.7	3.1	2.7
17:0	1.16 ± 0.24	tr[e]	tr	0.3
18:0	10.51 ± 1.14	21.3	15.2	16.0
18:1	21.46 ± 5.51	15.0	11.6	14.9
18:2	37.38 ± 1.96	30.6	39.2	45.4
18:3	1.02 ± 0.90	8.1	6.3	tr
19:0	3.50 ± 0.70	7.1	7.4	7.4
20:1	0.15 ± 0.05	tr	tr	tr
21:0	0.44 ± 0.12	tr	tr	tr
20:4	1.25 ± 0.07	tr	tr	tr
s/u[f]	0.7	0.78	0.67	0.59

[a]Results are expressed as the mean ± SD (n = 5).

[b,c,d]Values represent the averages of duplicate determinations from two similar experiments.

[e]tr, traces, amounts less than 0.2%.

[f]s/u, ratio of saturated to unsaturated fatty acids.

observed in some protozoa (27). Serine has been shown to be a good lipid precursor in *T. cruzi* (28). A decarboxylase may also be present in this organism, as was suggested for *C. fasciculata* (22). The increased incorporation of ^{14}C into lipids, when the hydroxymethyl carbon is specifically labeled, suggests that in *T. cruzi* transmethylation may play a role in phospholipid synthesis (28).

The upper phase of the lipid extraction was used for the isolation and identification of inositol phosphates by paper chromatography using n-propanol/concentrated ammonia/water (5:4:1, v/v/v) for developing. Our unlabeled standard was inositol 2-monophosphate which has an R_f value equal to that of inositol 4-phosphate (0.44). We therefore, could not distinguish the two. The inositol phosphate ratios observed for IP, IP_2 and IP_3 in *T. cruzi* were 1:1.24:1.67, respectively. Glycerophosphate and glycerophosphoinositol were identified by their R_f values of 0.53 and 0.61, respectively, as reported by Lapetina and Siess (29).

Some significant changes were observed in the relative percentages of fatty acid composition of the total lipids from *T. cruzi* (Table 2) compared to those found by others in epimastigote forms (29–31). *T. cruzi* total lipids contain only a small amount of arachidonic acid (1.5%). Major components were C_{18} and C_{16} acids as in *Leishmania donovani* (32). The major esterified fatty acids of PI, PIP and PIP_2 were different from those of mammalian phosphoinositides but similar to those of leishmania (26). In *T. cruzi*, however, relative increases in the C_{18} acids (except 18:2) and relative decreases in C_{16} acids were observed. The absence of arachidonic acid in these phospholipids (PIP and PIP_2) and its importance as a second messenger (33) and activator of protein kinase C (PKC) (34) might suggest that other unsaturated fatty acids may be able to replace arachidonic acid in certain of its roles. Moreover, several other unsaturated fatty acids, such as oleic acid and linoleic acid (35), are also effective stimulators of PKC. Wooten and Wrenn (36) reported that in pancreatic acinar cells linolenic acid activates this enzyme much more effectively than arachidonic or linolenic acid. Assuming that this is correct, this would point at the potential importance of some unsaturated fatty acids other than arachidonic acid in this type of organism.

LysoPC is both a stimulator of the kinase at low concentration and an inhibitor at high concentrations when acting together with Ca^{2+} and phosphatidylserine (PS) or Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DAG) (37). LysoPC-mediated activation of PKC is synergistic with that of DAG, but additive to that of oleic acid. Since the PC content of membranes is higher than that of PI, the potential capacity of the cells to generate free fatty acids and lysoPC is considerably greater than that for DAG.

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Effects of Dietary n-3 Fatty Acid-Enriched Chicken Eggs on Plasma and Tissue Cholesterol and Fatty Acid Composition of Rats

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The purpose of this study was to examine the effects of feeding n-3 polyunsaturated fatty acid (PUFA)-enriched chicken eggs on plasma and liver cholesterol levels and fatty acid composition in rats. Eggs were collected from laying hens fed diets containing 10% flax seed (Hn-3), 12% sunflower seed (Hn-6), or wheat and soybean meal control (CON). Yolk powders were prepared and fed at the 15% level to weanling female Sprague-Dawley rats for 28 days. Consumption of n-3 PUFA-enriched yolks significantly reduced both plasma and liver total cholesterol. Liver total lipids and phospholipids of rats fed Hn-3 diet were enriched with linolenic, eicosapentaenoic, and docosahexaenoic acids with a concomitant reduction of arachidonic acid in liver phospholipids. The plasma cholesterol of rats fed yolk powders enriched with n-6 PUFA (mainly linoleic acid) was reduced to the same extent as in those fed the n-3 enriched, but the liver cholesterol was significantly increased, indicating differential effects of dietary n-3 and n-6 PUFA. The results demonstrated that the cholesterolemic and tissue lipid modulating properties of chicken eggs could be modified in a favorable way by altering the fatty acid composition of yolk lipids through manipulation of laying hen diets.
Lipids 27, 279–284 (1992).

The past decade has witnessed a burst of interest in dietary n-3 polyunsaturated fatty acids (PUFA) and a growing consensus that consumption of n-3 PUFA is associated with a reduced incidence of coronary heart disease (1,2). α-Linolenic acid (LNA), the major plant n-3 fatty acid, exhibited beneficial effects similar to those of the longer chain n-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that occur in fish oil (3–6). It was thus suggested that 800 to 1100 mg per day of LNA and 300 to 400 mg per day of a combination of EPA and DHA are needed to prevent n-3 fatty acid deficiency in the elderly (7). Recently, the Canadian government adopted a recommendation that both n-6 and n-3 PUFA are essential nutrients and that the dietary supply should be at least 3 and 0.5%, respectively, of energy intake (8). The absolute amount of dietary n-3 fatty acids should be increased, and the ratio of n-6 to n-3 PUFA be reduced (9,10).

An alternate way to provide n-3 PUFA is by enriching the lipid components of animal products with n-3 PUFA since a major proportion of dietary fat in the Western world is of animal origin. The chicken egg appears to be quite suitable for this purpose (11,12). Preliminary studies from our laboratory have demonstrated that feeding full-fat flax seeds to laying hens greatly increased n-3 PUFA of the yolk (13,14). No studies, however, have reported the dietary effects of the n-3 PUFA-enriched eggs on lipid metabolism.

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Abbreviations: AA, arachidonic acid; CON, control diet; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; Hn-3, diet high in n-3 PUFA; Hn-6, diet high in n-6 PUFA; LA, linoleic acid; LNA, α-linolenic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TC, total cholesterol.

The present study was conducted to examine the lipid modulating property of n-3 PUFA-enriched eggs which were collected from laying hens fed diets containing flax seed. Eggs collected from laying hens fed a diet containing sunflower seed, and thus enriched with n-6 PUFA, were also included for comparison.

MATERIALS AND METHODS

Laying hen diets and yolk powder preparation. Single Comb White Leghorn pullets, 22 wk of age, were transferred to the test diets (Table 1), and the lighting was increased from 8 to 13 hr per day for the first wk, to 14 hr per day during the second wk, and maintained at 14 hr per day thereafter. The laying hen test diets containing 10% full-fat flax seed, 12% sunflower seed, or soybean meal plus animal tallow were provided *ad libitum*. The fatty acid composition of eggs was monitored, and the changes in fatty acid composition of the yolks were found to reach a plateau by 2 wk after feeding the test diets (data not shown). Ten dozen eggs laid during the sixth week were then collected from each treatment. The eggs were hard-boiled, and the yolks were separated, pulverized and dried at room temperature. Yolk powders thus obtained contained 96.3% dry matter. The fatty acid compositions of the yolk powder preparations were determined by gas chromatography (Table 2).

Animals and diets. Twenty-one female Sprague-Dawley rats, 4 wk of age, were housed individually in metabolic wire cages in a room with controlled temperature (21°C), humidity (60%) and lighting (12-hr light-dark cycle). Water and feed were provided *ad libitum*. After feeding a commercial diet (Wayne Rodent Blox, Continental Grain Company, Chicago, IL) for three days, they were divided into three groups with seven rats each, in such a manner that the mean body weight of each group was similar.

Three rat test diets were prepared by mixing 85% basal mix with 15% egg yolk powders (Table 3). The test diets contained yolk powders prepared from eggs laid by laying hens fed rations containing 10% flax seed (Hn-3, diet high in n-3 PUFA), 12% sunflower seed (Hn-6, diet high in n-6 PUFA), or soybean meal plus animal tallow (CON). The diets were stored at 4°C in dark plastic bags. Fresh feed was provided every two days, and feed consumption and body weight gain were measured weekly.

Sample preparation and analyses. At the end of the 28-day feeding trial, rats were fasted overnight and sacrificed by decapitation the following morning (09:00 to 11:00 hr). Trunk blood was collected into Na^+-heparin coated tubes. Rat liver tissue was quickly excised, blotted dry, weighed, and stored at −20°C for lipid analyses. Plasma was separated by centrifugation at 2,000 × *g* for 30 min. Plasma total cholesterol (TC) content was measured by an enzymatic method (15).

A portion (1.0 g) of the median lobe of rat liver was homogenized with a mixture of $CHCl_3$ and CH_3OH (2:1, v/v) (16) with 2 mg 5-α-cholestane added as an internal standard, and the liver total lipid content was determined gravimetrically. Liver cholesterol was determined

TABLE 1

Compositions of Laying Hen Test Diets

Ingredients	Laying hen test diets		
	10% Flax	12% Sunflower seed	Control
		%	
Wheat	64.66	62.21	68.69
Soybean meal	10.28	12.01	14.38
Flax seed	10.00	0	0
Sunflower seed	0	12.00	0
Animal tallow	3.25	2.00	5.08
Limestone	8.33	8.34	8.35
Dicalcium phosphate	1.00	0.98	1.01
Salt	0.29	0.29	0.28
DL-Methionine	0.09	0.07	0.09
Layer premix[a]	2.10	2.10	2.10
Calculated analyes			
Crude protein (%)	16.5	16.5	16.5
ME (Kcal/kg)	2700	2747	2700

[a]Supplied per kilogram diet the following: vitamin A, 8,000 IU; vitamin D_3, 1,200 ICU; vitamin E, 5 IU; riboflavin, 4 mg; calcium pantothenate, 6 mg; niacin, 15 mg; vitamin B_{12}, 10 μg; choline chloride, 100 mg; biotin, 100 μg; DL-methionine, 500 mg; selenium, 100 μg; manganese, 146 mg; zinc, 58 mg.

TABLE 2

Major Fatty Acids of Yolk Powders

Fatty acid	Yolk powders[a]		
	Hn-3	Hn-6	CON
	Total fatty acids (%)		
16:0	23.7	25.2	23.0
16:1	3.4	2.8	3.2
18:0	8.5	9.0	9.1
18:1	43.8	36.6	51.4
18:2n-6	10.6	21.8	8.8
18:3n-3	5.5	0.5	0.2
20:4n-6	0.8	2.2	1.8
20:5n-3	0.2	0	0
22:6n-3	1.9	0.6	0.8
Σn-3	7.9	1.1	1.3
Σn-6	11.4	24.3	10.7
18:2n-6 /18:3n-3	1.9	43.6	44.0
Σn-6/Σn-3	1.4	22.1	8.2

[a]Yolk powders were prepared from eggs of laying hens fed diets containing 10% full-fat flax (Hn-3), 12% sunflower seed (Hn-6), or soybean meal plus animal tallow (CON).

by gas chromatography as described previously (17). Aliquots of the liver lipid extracts were dried under nitrogen and converted to their methyl esters by incubation with a mixture of BF_3/methanol (10% BF_3 in methanol), hexane and methanol (35:20:45, v/v/v) at 90°C for 30 min (18). Liver phospholipids were separated on silica gel G plates (Sigma Chemical Co., St. Louis, MO) by developing the plates in a solvent mixture of hexane, diethyl ether and acetic acid (80:20:1, v/v/v) for 30 min. The regions corresponding to the phospholipid standards were scraped into screw-capped tubes and methylated directly in the same way as for the liver total lipids. The methyl esters of fatty acids of liver total lipids and phospholipids were separated and quantified by gas chromatography equipped with an on-column injector (Varian 3400, Varian Associates Inc., Sunnyvale, CA) using a DB-23 fused capillary column (30 m × 0.25 mm i.d.) (J & W Scientific, Folsom, CA). A two-stage column temperature program was used. The initial temperature was set at 70°C for 0.3 min, increased to 180°C at 30 C/min and held for 10 min. Then the column temperature was elevated to 230°C at a rate of 5°C/min and held at the final temperature for 3 min. Helium was used as the carrier gas. The flame ionization detector was set at 240°C.

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA). Treatment means were further analyzed by Duncan's multiple range test (19).

RESULTS

Fatty acid profiles of yolk lipids. The fatty acid composition of the yolk powders was readily modified by the laying hen test diets (Table 2). Yolk powders from laying hens fed diets containing flax seed contained 6.1 times the total n-3 PUFA of the control. LNA was the major n-3 PUFA deposited in Hn-3 yolks, although considerable amounts of EPA and DHA were also found in these yolks. Feeding flax seed to laying hens also slightly increased yolk LA and decreased yolk arachidonic acid (AA) content. On the other hand, yolk powder from laying hens fed sunflower seed diet more than doubled the n-6 linoleic acid (LA) in place of oleic acid. Consequently, the ratios of LA/LNA and Σn-6/Σn-3 PUFA were dramatically decreased in Hn-3 yolk powders, and increased in Hn-6 yolk powder preparation, when compared to the control.

Rat performance and organ weights. Rat test diets did not affect the daily feed consumption or body weight gain, nor the absolute weight or the percentage of body weight of liver and heart tissues (Table 4), indicating that enrichment of yolks with either n-3 or n-6 PUFA did not adversely affect the overall feeding values of the yolk.

TABLE 3

Composition and Proximate Analyses of Rat Test Diets

Ingredient[a]	g/kg Feed	Hn-3	Hn-6	CON
Casein, 87.3% CP	175.0			
Yolk powder[b], 34.5% CP	150.0			
Corn starch	360.0			
Glucose, monohydrate	200.0			
DL-Methionine	2.6			
Choline chloride	1.8			
Inositol	5.6			
Vitamin mix, A.O.A.C.	10.0			
Mineral mix, Bernhart-Tomarelli	45.0			
Cellulose	50.0			
Proximate analyses of the test diets[b]				
Protein (%)		20.1	20.0	19.7
Total lipid (%)		9.9	10.0	10.3
Cholesterol (%)		0.37	0.37	0.39

[a]All nutrients, except yolk powder, were purchased from Teklad, Madison, WI.

[b]Test diets used yolk powders prepared from eggs laid by hens fed diets containing 10% flax seed (Hn-3), 12% sunflower seed (Hn-6), or soybean meal plus animal tallow (CON).

TABLE 4

Initial Body Weight, Daily Body Weight Gain and Feed Consumption, Liver and Heart Weights of Rats Fed Test Diets

Diet[a]	Initial wt (g)	Net gain (g/rat/day)	Feed consumption (g/rat/day)	Liver wt (g)	Heart wt (g)
Hn-3	80.3	4.6	16.5	7.08	0.91
Hn-6	82.7	4.6	16.6	7.71	0.89
CON	82.7	4.4	16.7	7.21	0.86
SEM	2.19	0.13	0.45	0.27	0.04

[a]The test diets used yolk powders prepared from eggs of laying hens fed diets containing 10% full-fat flax (Hn-3), 12% sunflower seed (Hn-6), or soybean meal plus animal tallow (CON). Female Sprague-Dawley rats were fed test diets for a period of 28 days (n = 7).

Plasma and liver total cholesterol levels. Feeding the Hn-3 diet significantly reduced plasma total cholesterol (TC) as well as liver cholesterol content in rats (Fig. 1). On the other hand, the Hn-6 diet reduced plasma TC level to the same extent as did the Hn-3 diet, but elevated hepatic cholesterol concentration significantly. As the weight of the rat liver was not affected by dietary treatments (Table 4), the Hn-3 diet thus significantly reduced the hepatic cholesterol pool while the Hn-6 augmented this pool when compared to the control diet. Liver total lipid content was also highest in rats fed the Hn-6 diet (Fig. 1).

Fatty acids of liver total lipids and phospholipids. The fatty acids of liver total lipids were changed by rat test diets (Table 5). Significantly higher contents of LNA and its metabolites EPA, docosapentaenoic acid (DPA), and DHA, were found in liver total lipids of rats fed Hn-3 than those fed the control diet (CON). In rats fed the n-6 PUFA-enriched yolk powder diet, neither total nor individual n-3 PUFA were changed when compared to the control fed group. The Hn-6 diet, although it increased LA content significantly, had no effect on the AA level of liver total lipids.

The LNA content of liver phospholipids was not affected by dietary treatments (Table 6). EPA in liver phospholipids of rats fed the Hn-3 diet was 0.9% of total fatty acids, a significant increase over those in Hn-6 or CON fed rats. Hn-3 feeding compared to CON feeding also increased DHA and reduced AA contents significantly in liver phospholipid fraction. Slightly higher LA content found in the liver phospholipids of rats fed the Hn-3 diet might be the result of a slightly higher LA content in the Hn-3 yolks (Table 2). The Hn-6 diet increased the LA content of liver phospholipids, but did not elevate the AA content. When compared to the liver total lipids, liver phospholipids were particularly rich in longer chain metabolites of LA and LNA, such as AA, EPA, and DHA.

DISCUSSION

Since n-3 fatty acids play important roles in human health and disease, and particularly in cardiovascular disease, major nutritional advisory groups have recommended to increase the intake of n-3 fatty acids and to lower the dietary n-6 to n-3 fatty acid ratio (7–10). In the present study, we employed chicken eggs to provide the much

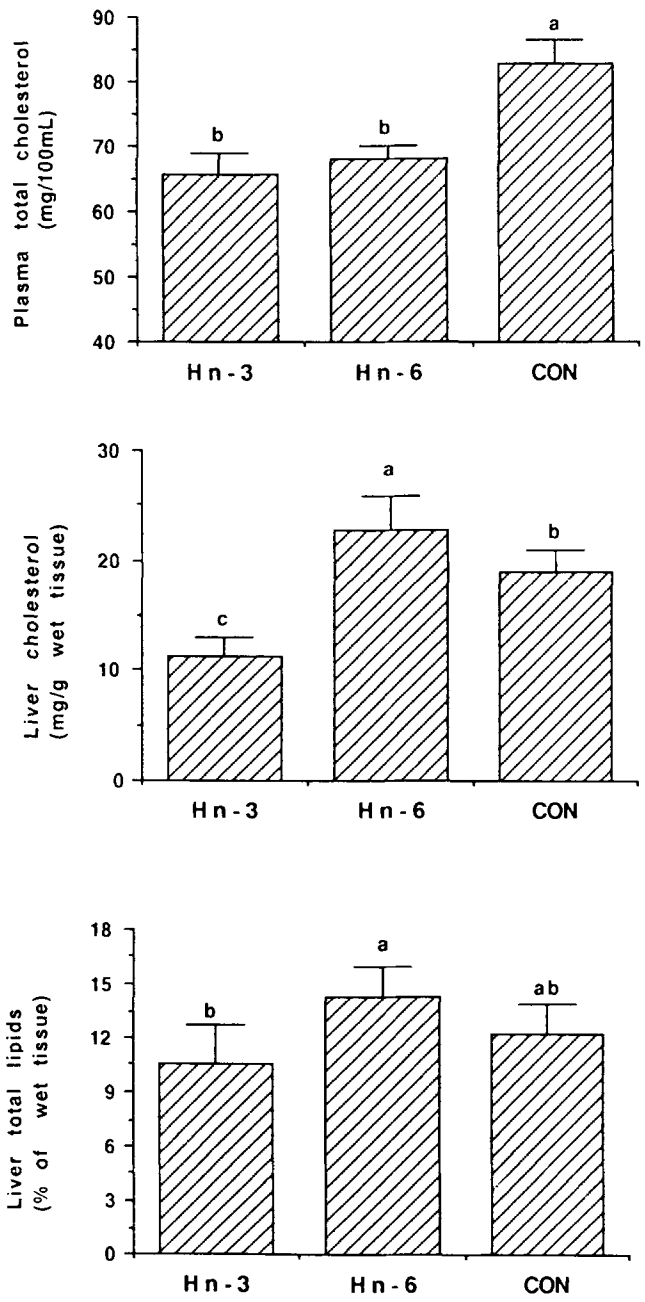

FIG. 1. Plasma and liver cholesterol levels and liver total lipid content of rats at the end of 28-day feeding trial (mean ± SEM, n = 7). The test diets used yolk powders prepared from eggs laid by laying hens fed diets containing 10% flax seed (Hn-3), 12% sunflower seed (Hn-6), or animal tallow control (CON). Bars without a common letter differ significantly ($P < 0.05$).

needed n-3 PUFA and to lower the n-6 to n-3 ratio. A large egg from laying hens fed the 10% flax diet, for example, provided about 450 mg of n-3 PUFA, one-third of which was due to longer chain n-3 PUFA such as EPA, DPA, and DHA. Furthermore, the n-6 to n-3 ratio was reduced from 8.2 in regular eggs to 1.4 in n-3 PUFA-enriched eggs.

The intake of n-3 PUFA-enriched eggs reduced not only the plasma total cholesterol level, but also the liver cholesterol content. In contrast, while the plasma cholesterol content of rats was reduced to the same extent as in those fed the Hn-3 diet, Hn-6 feeding resulted in the accumulation of more cholesterol in rat liver tissue. Thus eggs enriched with n-3 PUFA were more potent than those enriched with n-6 PUFA in reducing the body cholesterol pool. This discrepancy was attributable to the different effects of dietary LNA and LA on cholesterol metabolism in rats. The accumulation of cholesterol in hepatic tissue was also observed in rats fed vegetable oils rich in LA (3,20,21).

The increase of n-3 or n-6 PUFA in yolks was in place of monounsaturated fatty acids (MUFA, Table 2). A hypocholesterolemic effect of dietary MUFA was reported in human subjects (22). In the present study, however, replacement of MUFA by n-3 and n-6 PUFA resulted in a significant reduction in plasma cholesterol level, indicating that dietary n-3 or n-6 PUFA were more potent than dietary MUFA in lowering plasma cholesterol in rats. Previous studies have also revealed that substitution of MUFA for saturated or n-6 PUFA resulted in higher plasma and liver cholesterol levels in rats (23–26).

When dietary n-3 PUFA were provided exclusively as LNA from vegetable oils such as flax and canola oils, a concentration higher than 12% of total fatty acids was needed to effect a lower plasma cholesterol in rats (3,4,27). In the present study, the Hn-3 diet contained 7.9% total n-3 PUFA but still lowered plasma and liver cholesterol significantly. The n-3 PUFA-enriched yolks contained 2.4% EPA, DPA, and DHA in addition to 5.5% LNA, while flax, soybean and canola oils contained LNA as the only n-3 PUFA. The longer chain n-3 PUFA (EPA and DHA) might be more potent than LNA in lowering plasma cholesterol (28,29). In addition, the major portion of the longer chain n-3 PUFA (EPA, DPA, and DHA) in the n-3 yolks resided in phospholipid classes, particularly in phosphatidylethanolamine (30). Dietary phospholipids were reported to have a specific and systemic influence on cholesterol metabolism (31,32), and the degree of the unsaturation of dietary phospholipids directly influenced rat plasma cholesterol levels (32). The enrichment of yolk phospholipids with EPA, DPA, and DHA might thus enhance the ability of lipid modification by n-3 PUFA-enriched yolks. Furthermore, female rats were used in this study. The plasma cholesterol of female rats was more responsive to dietary manipulation than that of male ones (33,34).

The consumption of the n-3 PUFA-enriched yolks not only diminished the body cholesterol pool, but more importantly enriched liver total lipids and phospholipids with LNA, EPA, DPA, and DHA (Table 5,6). In addition, the AA content of liver phospholipids was reduced. The reduction of AA content could be attributable to the inhibition of AA synthesis by the n-3 PUFA in the Hn-3 diet and/or a lower dietary AA content of Hn-3 yolks. Both the enrichment with n-3 PUFA and the reduction of the AA content in tissue phospholipids are considered to have ameliorating effects on atherosclerosis (2).

When rats were fed n-6 PUFA-enriched yolk powders, the LA content of liver total lipids and phospholipids increased, whereas the AA content was not affected. In general, increasing the dietary intake of LA tended to elevate tissue AA content. However, often no further increase of tissue AA content was observed due to the de-

TABLE 5

Major n-3 and n-6 Fatty Acids of Rat Liver at the End of the Feeding Trial[a]

Fatty acid	Dietary treatment		
	Hn-3	Hn-6	CON
	Total fatty acids (%)		
18:2n-6	9.5 ± 0.5[c]	13.8 ± 0.9[b]	8.0 ± 0.3[d]
20:4n-6	8.8 ± 1.1[b]	9.6 ± 1.4[b]	9.7 ± 0.2[b]
18:3n-3	1.8 ± 0.3[b]	0.3 ± 0[c]	0.3 ± 0[c]
20:5n-3	1.1 ± 0.3[b]	0.1 ± 0[c]	0.2 ± 0[c]
22:5n-3	0.5 ± 0.1[b]	0.1 ± 0[c]	0.1 ± 0[c]
22:6n-3	4.8 ± 0.8[b]	2.0 ± 0.3[c]	2.4 ± 0.4[c]

[a]Data were presented as mean ± SEM, n = 7. The test diets used yolk powders prepared from eggs of laying hens fed diets containing 10% full-fat flax (Hn-3), 12% sunflower seed (Hn-6), or soybean meal plus animal tallow (CON). Means in a row without common superscripts (b-d) differ significantly ($P < 0.05$).

TABLE 6

Major Fatty Acids of Liver Phospholipids in Rats Fed Test Diets[a]

Fatty acid	Dietary treatment		
	Hn-3	Hn-6	CON
	Total fatty acids (%)		
16:0	16.4 ± 1.4[b,c]	17.1 ± 0.7[c]	15.7 ± 0.6[c]
18:0	27.5 ± 1.9[b]	26.6 ± 1.7[b]	27.5 ± 1.6[b]
18:1	15.0 ± 3.0[b]	16.7 ± 2.5[b]	15.9 ± 2.3[b]
18:2n-6	7.5 ± 0.9[b]	7.8 ± 0.6[b]	6.2 ± 1.0[c]
18:3n-3	0.4 ± 0.3[b]	0.5 ± 0.3[b]	0.3 ± 0.3[b]
20:4n-6	19.8 ± 2.9[c]	21.2 ± 2.1[b,c]	24.1 ± 2.3[b]
20:5n-3	0.9 ± 0.3[b]	0	0
22:6n-3	7.9 ± 1.2[b]	5.0 ± 1.1[c]	5.6 ± 0.8[c]
20:4n-6/ (20:5 + 22:6)n-3	2.25	4.24	4.30

[a]Data were presented as mean ± SEM, n = 7. The test diets used yolk powders prepared from eggs of laying hens fed diets containing 10% full-fat flax (Hn-3), 12% sunflower seed (Hn-6), or soybean meal plus animal tallow (CON). Means in a row without common superscripts (b-d) differ significantly ($P < 0.05$).

crease of tissue Δ6 desaturase activity when diet was rich in LA (3,35,36).

In conclusion, the cholesterolemic and lipid modulating properties of dietary egg yolks could be modified by altering the fatty acid composition of yolk lipids through nutritional manipulation of laying hens. The n-3 PUFA-enriched animal products (eggs and meats) could become an important dietary source of nutritionally important n-3 PUFA. Caution, however, must be exercised in extrapolating the rat data to man. Further studies with human subjects appear therefore warranted.

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METHOD

Effects of *bis* Homoallylic and Homoallylic Hydroxyl Substitution on the Olefinic ^{13}C Resonance Shifts in Fatty Acid Methyl Esters[1]

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Substitution of a hydroxyl group at the *bis* homoallylic position (OH group located three carbons away from the olefinic carbon) in C_{18} unsaturated fatty acid esters (FAE) induces a 0.73 ± 0.05 ppm upfield and a 0.73 ± 0.06 ppm downfield shift on the δ and ε olefinic ^{13}C resonances relative to the unsubstituted FAE, respectively. If the hydroxyl group is located on the carboxyl side of the double bond of the *bis* homoallylic hydroxy fatty acid esters (BHAHFA), the olefinic resonances are uniformly shifted apart by [|1.46+|Δδdb$_u$||] where Δδdb$_u$ represents the absolute value of the double bond resonance separation in the unsubstituted FAE and 1.46 ppm is the sum of the absolute values of the δ and ε shift parameters. With hydroxyl substitution on the terminal methyl side of the double bond, the olefinic shift separation is equal to [|1.46−|Δδdb$_u$||]. In homoallylic (OH group located two carbons away from the olefinic carbon) substituted FAE the γ and δ induced hydroxyl shifts for the *cis* double bond resonances are +3.08 and −4.63 ppm, respectively while the *trans* double bond parameters are +4.06 and −4.18 ppm, respectively. The double bond resonance separation in homoallylic hydroxy fatty acid esters (HAHFA) can be calculated from the formula [|7.71−|Δδdb$_u$||] for *cis* and [|8.24−|Δδdb$_u$||] for the *trans* case when the OH substitution is on the carboxyl side of the double bond. Conversely, when the OH resides on the terminal methyl side, the double bond shift separations for *cis* and *trans* isomers are [|7.71+|Δδdb$_u$||] and [|8.24+|Δδdb$_u$||], respectively. The derived shift parameters can verify the positions of both the double bond and hydroxyl substitution from the olefinic resonance separation in long-chain fatty acid derivatives, obviating the need for destructive analytical methods.
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Although there are a number of articles concerning the ^{13}C nuclear magnetic resonance (NMR) assignments of fatty acid structures (1,2) including unsaturated fatty acids (3–6) and hydroxy fatty acids (7), no attempts have been made to systematize the ^{13}C NMR assignments of unsaturated hydroxy fatty acids. Spectral assignments for methyl ricinoleate, methyl ricinelaidate, and homoallylic hydroxy fatty acid esters (HAHFA) have been mentioned by Rakoff *et al.* (8) and so have assignments of methyl 12-hydroxy-10-octadecenoate by Frankel *et al.* (9).

[1] Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

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Abbreviations: BHAFA, *bis* homoallylic fatty acid esters; FAE, fatty acid esters; HAHFA, homoallylic fatty acid esters; NMR, nuclear magnetic resonance; THP, tetrahydropyranyl.

Earlier work by Tulloch and Mazurek (7) demonstrated that the position of hydroxylation in saturated fatty acids could be pinpointed by examining the induced shifts in natural abundance ^{13}C spectra of the hydroxylated and adjacent carbon resonances. In these spectra the carbon carrying the hydroxyl group (α carbon) was found to shift 42.2 ppm downfield, the β carbon downfield 7.80 ppm, and γ and δ shifted upfield 4.0 and 0.6 ppm, respectively. Batchelor *et al.* (5) have undertaken an extensive investigation of the electric field effects of the carboxyl group on the ^{13}C double bond resonances and of the effects of other substituents such as cyclopropane and additional double bonds on saturated and unsaturated carbon resonances in long-chain fatty acid derivatives (1). Their findings demonstrated that of the two unsaturated carbons, the carbon nearest the carboxylate head group consistently had the higher field ^{13}C chemical shift. It was also revealed that the separation of the double bond ^{13}C resonances uniquely depends upon the position of the double bond relative to the carboxyl group. These observations were attributed to a linear electric field effect induced by the terminal carboxyl group. In the present work we report the results of hydroxyl substitution on the chemical shift separation of unsaturated carbon resonances in long-chain fatty acid esters together with some strategies for exploiting derived shift parameters for structural identification of these derivatives.

MATERIALS AND METHODS

Materials. Unsaturated and hydroxy-substituted fatty acids and esters such as those of petroselinic, ricinoleic, ricinelaidic, oleic, and elaidic acids were obtained commercially. Most of the ^{13}C NMR data were taken from various literature sources (3,4,7–9). Chemical shift assignments for the unsaturated carbon resonances in *cis* and *trans* HAHFA, methyl 12-OH-9-octadecenoate, were obtained from Rakoff *et al.* (8). Methyl 9-hydroxy-12-octadecenoate (isoricinoleate) was obtained by extracting seeds of *Holarrhena antidysenterica* (NU-46607) and purification as described (10).

NMR spectroscopy. ^{13}C NMR spectra were obtained with a 9.3T JEOL GX-400 NMR spectrometer (Peabody, MA) operating at 100.4 MHz. All spectra were obtained with a 25KHz spectral width, 16K data points, a 15 μsec 90 degree pulse, and a 5 sec repetition rate. Samples were examined in $CDCl_3$. All resonances were referenced to $CDCl_3$ at 77.00 ppm relative to external TMS which is assigned a value of 0.00 ppm. Reported chemical shifts are ±0.03 ppm.

Mass spectrometry. Mass spectra were obtained on a Hewlett-Packard 5990B GC/MS fitted with an Ultra (methyl silicone) 12-m capillary column (Hewlett-Packard,

Palo Alto, CA). The column was temperature programmed from 150° to 250°C at 4°C per min.

Methyl 10-hydroxy-6-octadecenoate and methyl 9-hydroxy-5-octadecenoate were synthesized by the following procedure: The synthesis of the δ-6,7-structure began with 5-chloro-1-pentanol that was protected as a tetrahydropyranyl (THP) ether, and was then treated with lithium acetylide in dimethyl sulfoxide (iodide catalyst). The resulting alkyne was cyanoethylated (1:butyllithium, ethylene oxide; 2:methanesulfonyl chloride, triethylamine, 3:sodium cyanide, dimethyl sulfoxide), and the resulting nitrile was then condensed with n-octylmagnesium bromide to produce a ketoalkyne of the correct chain length bearing a THP-protected primary alcohol. Deprotection (methanolic HCl) gave a ketoalkynol that was oxidized to the acid (chromic acid, acetone) and esterified (methanol, boron trifluoride). This ketoalkyne ester was reduced at the carbonyl by sodium borohydride and hydrogenated to a *cis*-alkene over palladium on carbon in methanol. The δ-5,6-structure was synthesized in analogous fashion using 4-chloro-1-butanol and 1-bromononane as alternate starting materials. The positions of the hydroxyl group and double bonds were verified by oxidative cleavage and mass spectrometry. Double bond position was determined by the chromic acid micro column technique (11). The scission products (carboxylic acids) were identified by comparison of retention times with standards using gas-liquid chromatography, and also by mass spectrometry. The hydroxyl group was localized by mass spectrometry after hydrogenation of the double bond (12).

RESULTS AND DISCUSSION

Figure 1 gives a schematic representation of the two types of unsaturated hydroxy fatty acids examined in this study. Each of these two structures, *bis* homoallylic (BHAHFA), OH located three carbons away from the olefinic carbon and homoallylic (HAHFA), OH located 2 carbons away from the olefinic carbon fatty acid esters, can contain a hydroxyl substituent on the terminal methyl side [a] or carboxyl side [b] of the double bond. Table 1 lists as an example the observed ^{13}C chemical shifts for the newly synthesized BHAHFA II methyl 9-OH-*cis*-5-octadecenoate, methyl 10-OH-*cis*-6-octadecenoate as well as the previously isolated methyl 9-OH-*cis*-12-octadecenoate (isoricinoleate) and the corresponding unsaturated methyl esters from which they are derived (3). The incremental ^{13}C shifts due to the substitution of the hydroxyl group

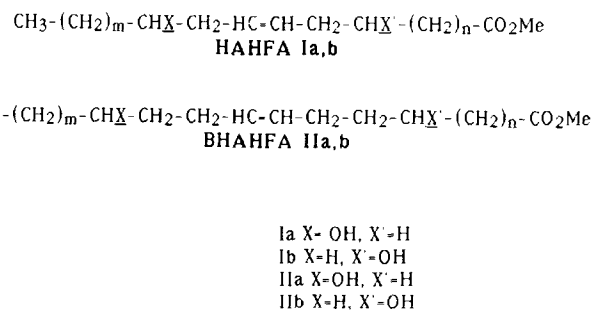

FIG. 1. Schematic representation of monounsaturated hydroxy fatty acid methyl ester structures.

TABLE 1

Chemical Shifts[a] of the Monounsaturated Fatty Acid Methyl Esters and BHAHFA Type IIa and b Fatty Acid Methyl Esters

Compound name	Carbon number																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Methyl *cis*-12-octadecenoate	174.35	34.20	25.10	29.30	29.40	29.55	29.65	29.60	29.40	29.85	27.30	130.00	130.00	27.30	29.55	31.65	22.70	14.1
9-OH substitution increment					−0.16	−.06	−4.06	7.71	42.31	7.61	−3.70	−0.81	0.69					
Methyl 9-OH-*cis*-12-octadecenoate	174.33	34.11	25.94	29.10	29.24	29.61	25.5	37.36	71.71	37.46	23.60	129.19	130.69	27.23	29.41	31.56	22.60	14.09
Methyl *cis*-6-octadecenoate	173.80	33.97	24.60	29.21	26.83	128.98	130.28	27.22	29.72	29.33	29.56	29.66	29.66	29.66	29.33	31.92	22.69	14.09
10-OH substitution increment					0.87	−0.63	−3.69	7.82	42.10	7.66	−4.02	0.04	−0.08					
Methyl 10-OH-*cis*-6-octadecenoate	174.15	33.89	24.49	29.06	26.75	129.85	129.65	23.53	37.54	71.43	37.22	25.64	29.70	29.58	29.24	31.85	22.62	14.04
Methyl *cis*-5-octadecenoate[b]	174.25	33.55	25.00	26.65	128.40	131.30	27.35	29.75	29.45	29.65	29.75	29.75	29.75	29.65	29.45	32.00	22.75	14.15
9-OH substitution increment					0.65	−0.75	−3.80	8.00	42.06	7.59	−4.05	−0.02	−0.08					
Methyl 9-OH-*cis*-5-octadecenoate	174.28	33.45	24.89	26.56	130.55	129.09	23.55	37.75	71.51	37.24	25.70	29.73	29.67	29.61	29.35	31.92	22.70	14.12

[a]ppm. [b]Reference 3.

are given for each structure. Note that the OH induced shifts in the BHAHFA associated with the ^{13}C resonances representing the adjacent saturated carbons are similar to those reported earlier (7). However, the double bond carbon resonances, those δ and ε to the OH substituted carbon, have undergone a relatively large shift change as compared to their saturated counterparts, presumably because of the greater polarizability of the unsaturated carbons by the electric field originating at the molecular dipole of the OH group (1,5).

Table 2 lists the observed OH induced shift parameters for the double bond carbon resonances in HAHFA Ia,b and BHAHFA IIa,b. The values represent the average difference in chemical shift between the olefinic carbon resonances in the substituted and non-substituted esters. By combining the shift separation values for the monounsaturated fatty acid methyl esters as given in Table 3 with the shift parameters in Table 2 we can predict the double bond resonance separation in type IIa and b BHAHFA (Fig. 2 and 3). Figure 2 illustrates when the OH group is on the terminal methyl side of the double bond, the double bond resonance separation shows a continuous narrowing as the double bond position moves from carbon 12 to 6. The narrowing in the olefinic shift separations results from the upfield movement (−0.73 ppm) of the lower field shift (δ carbon) and the downfield movement (+0.73 ppm) of the higher field olefinic resonance (ε carbon). Therefore, in type IIa structures with unsaturation in positions 6–12, the double bond resonance assignments

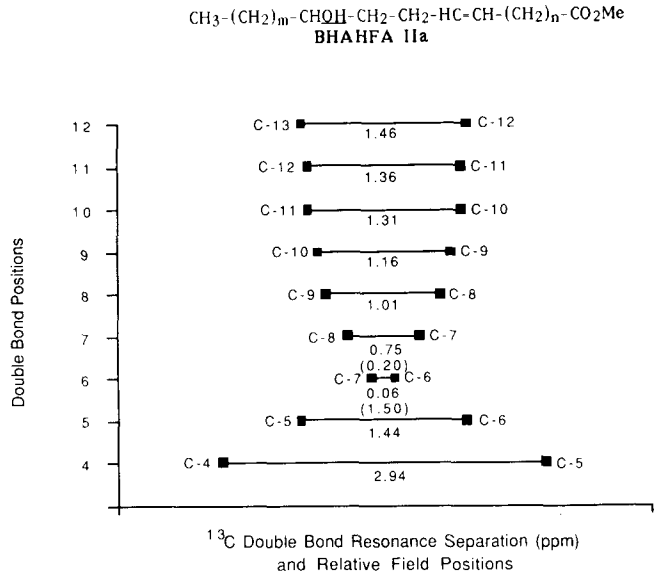

FIG. 2. Predicted and actual ^{13}C double bond resonance separations, chemical shift assignments and relative field positions for BHAHFA when the OH group is on the terminal methyl side of the double bond (type IIa). Value given in parentheses over the line is the actual shift separation value.

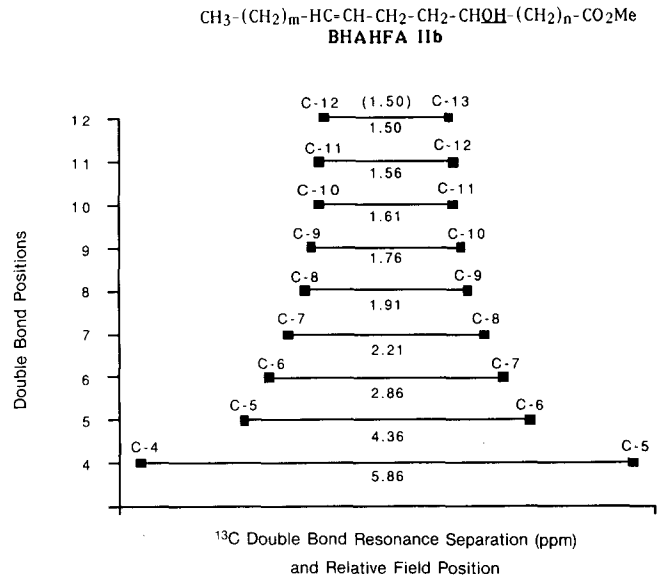

FIG. 3. Predicted and actual ^{13}C double bond resonance separations, chemical shift assignments and relative field positions for BHAHFA when the OH group is on the carboxyl side of the double bond (type IIb). Value given in parentheses over the line is the actual shift separation value.

TABLE 2

Hydroxyl Shift Parameters (ppm) for Substituted Fatty Acid Methyl Esters

Double bond position relative to OH	HAHFA		BHAHFA
	cis	*trans*	*cis*
γ	3.08[a]	4.06[a]	
δ	−4.63[a]	−4.18[a]	−0.73 ± 0.05
ε			0.73 ± 0.06

[a]Based on shifts for the *cis* and *trans* isomers of methyl 12-hydroxy-9-octadecenoate and methyl 10-hydroxy-*trans*-12-octadecenoate.

TABLE 3

Predicted ^{13}C Double Bond Chemical Shift Separations (ppm) in *cis* and *trans* HAHFA Ia and b Based on Induced Shift Parameters Given in Table 2

Double bond position	$\Delta\delta db_u$[a]		$\Delta\delta db_s$				
	cis	*trans*	*trans* Ia,b	*cis* Ia	*cis* Ib	*trans* Ia	*trans* Ib
12	0.00	0.00	1.15	7.71	7.71	8.24	8.24
11	0.10	0.10	1.25	7.61	7.81	8.14	8.34
10	0.15	0.15	1.30	7.56	7.86	8.09	8.39
9	0.30	0.25	1.40	7.41	8.01	8.01	8.49
8	0.45	0.45	1.60	7.26	8.16	7.79	8.69
7	0.75	0.75	1.90	6.96	8.46	7.49	8.99
6	1.40	1.45	2.60	6.31	9.11	6.79	9.69
5	2.90	2.90	4.05	4.81	10.61	5.34	11.14
4	4.40	4.40	5.55	3.31	12.11	3.84	12.64

[a]Based on shifts given by Bus *et al.* (4).

are opposite to those reported for the unsubstituted monounsaturated counterparts (5). That is, in type IIa compounds (double bond positions 6–12 and beyond) the higher field olefinic resonance represents the olefinic carbon farthest from the carboxyl group. At the 6-position the olefinic shift separation reaches a minimum (0.1 ppm) due to the countering dipolar effects of the terminal carboxyl group (5). Beyond the 6-position *e.g.*, double bond

positions 4, 5, etc. the higher and lower field olefinic resonance assignments revert to those previously established for the corresponding fatty esters (3). In essence, these shifts have crossed over each other.

In Figure 3 we observe that the predicted double bond shift assignments for type IIb compounds do not change from the corresponding unsubstituted precursors (4,5), since the upfield resonance (δ) moves upfield (−0.73 ppm) and the downfield resonance (ε) moves downfield (0.73) with OH substitution on the carboxyl side of the double bond. Since no shift crossovers can occur in this example, only a smooth increment in double bond resonance separation is observed as the double bond moves from C-12 to C-4.

To calculate the double bond shift separation in BHAHFA we use the following:

$$\text{For type IIa} \qquad \Delta\delta db_s = [||\varepsilon\text{-}\delta| + |\Delta\delta db_u||] \qquad [1]$$

Where $\Delta\delta db_s$ is the double bond resonance separation in the BHAHFA, $|\varepsilon\text{-}\delta|$ is 1.46 ppm for *cis* type II fatty acid esters and $\Delta\delta db_u$ is the observed ^{13}C shift separation in the monounsaturated analogues.

$$\text{For type IIb} \qquad \Delta\delta db_s = [||\varepsilon\text{-}\delta| - |\Delta\delta db_u||] \qquad [2]$$

HAHFA double bond resonance separations are given by:

$$\Delta\delta db_s = [||\gamma\text{-}\delta| - |\Delta\delta db_u||] \qquad [3]$$

For type Ia *cis* or *trans* where $|\gamma\text{-}\delta|$ is equal to 7.71 and 8.24 ppm for the *cis* and *trans* isomers, respectively (Table 2). For type Ib the same equation as above applies except the sign is positive:

$$\Delta\delta db_s = [||\gamma\text{-}\delta| + |\Delta\delta db_u||] \qquad [4]$$

Predicted shift separations for *cis* and *trans* type a or b HAHFA are given in Table 3. In most instances one can pinpoint the position of hydroxyl substitution in long-chain fatty acid derivatives directly from the documented α shift (7). Having established this position, it is easy to ascertain if a double bond is located within 3 or 2 carbons from this site as well as on which side of the double bond it resides. Note that it is not possible to determine if we have type a or b substitution in either BHAHFA or HAHFA derivatives when the double bond is at the 12-position since the chemical shifts are identical for the olefinic carbons in the spectra of the corresponding unsubstituted fatty acid esters (FAE). Also, we have not accounted for possible effects of H-bonding of the OH group with the carboxyl when OH substitution is found at the C-4 or position closer to the carboxyl group. ^{13}C chemical shift data available from the literature on allylic hydroxy FAE do not appear to show the same predictable shift increments exhibited by derivatives I and II.

The shift parameters described in this work can be useful for the non-destructive identification of structures of type Ia,b and IIa,b FAE. It is particularly valuable when sample size is limited and material cannot be sacrificed for destructive analyses. While alternative NMR methods such as 2D INADEQUATE experiments (13) are often used to elucidate such structures, they require orders of magnitude of more sample and time than is required for this simple analysis.

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Chemiluminescence Detection of 8a-Hydroperoxy-tocopherone in Photooxidized α-Tocopherol

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Photosensitized oxidation products of α-tocopherol were analyzed by reversed phase high-performance liquid chromatography (CL-HPLC) in combination with chemiluminescence detection, to measure hydroperoxide derivatives. An intense peak was observed on the chemiluminescence chromatogram, corresponding to 8a-hydroperoxy-α-tocopherone (α-tocopherol hydroperoxide), the identity of which was confirmed by ^1H nuclear magnetic resonance and mass spectrometry. Observed peak intensities correlated with the peroxide value of oxidized α-tocopherol. As little as 50 pmol of 8a-hydroperoxy-α-tocopherone was detectable. The CL-HPLC method, which is specific for the detection of hydroperoxides, should prove useful in studies on tocopherol oxidation in foods and biological systems.
Lipids 27, 289–294 (1992).

Tocopherol (Toc) has received much attention as a very important antioxidant both in foods and biological systems (1,2). Toc is known to react with singlet molecular oxygen ($^1O_2$) whereby $^1O_2$ is chemically and physically quenched by Toc (3,4). Toc-quinone and Toc-quinone epoxide have been reported to be formed by $^1O_2$ oxidation of Toc in several model systems (5–7). The occurrence of Toc-quinone in rat liver also has been reported (8,9). However, details of the oxidation mechanism of Toc with oxygen radicals and with electrically excited species of $^1O_2$ are not fully understood.

Clough *et al.* (3) suggested that $^1O_2$ oxidation of Toc takes place *via* formation of 8a-hydroperoxy-tocopherone (Toc-OOH) as an intermediate. Matsumoto *et al.* (10) demonstrated by X-ray analysis that the reaction of 2,2,5,7,8-pentamethyl-chroman-6-ol (a model compound for Toc) with $^1O_2$ gives rise to 8a-hydroperoxy-2,2,5,7,8-pentamethyl-chroman-6-one, which is an important precursor of Toc oxidation products. Recently, Yamauchi *et al.* (11) reported that 8a-hydroperoxy-α-tocopherone was generated by radical initiated oxidation of α-Toc. Hydroperoxidic Toc has been characterized by its mass spectrum, infrared spectrum, and by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. No evidence has been published on the occurrence of hydroperoxy-tocopherones in biological systems. An established method that would be specific for the detection of the hydroperoxide group of Toc-OOH is not presently available.

Miyazawa and co-workers (12–15) have described a chemiluminescence detection high-performance liquid chromatographic (CL-HPLC) method for the assay of phospholipid hydroperoxides present in plasma and tissues. In the present study, we have applied the CL-HPLC method for the assay of Toc-OOH formed during the reaction of α-Toc with photochemically-generated $^1O_2$. We observed the appearance of a chemiluminescence peak that we ascribed to Toc-OOH, and 8a-hydroperoxy-tocopherone was identified as the principal chemiluminescent product.

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Abbreviations: CL, chemiluminescence; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; $^1O_2$, singlet oxygen; TLC, thin-layer chromatography; Toc, Tocopherol; Toc-OOH, tocopherol hydroperoxides.

MATERIALS AND METHODS

CL-HPLC system. The CL-HPLC system used in the present study to detect Toc-OOH was essentially the same as that reported by Miyazawa (12). It consisted of a Reodyne model 7125 sample injector and a Wakopak NU-ODS-10 column (4.6 × 250 mm, packed with 10 μm Nucleosil-ODS-Si; Wako Pure Chemical Co., Osaka, Japan) coupled to a Jasco 875 UV detector (Japan Spectroscopic Co., Tokyo, Japan). Methanol was used as the mobile phase for HPLC at a flow rate of 1.1 mL/min, which was controlled by a Jasco 880 PU pump. Column eluate was first monitored at 240 nm by a UV detector. After UV detection, the column eluate was mixed with a chemiluminescence (CL) reagent consisting of cytochrome c (10 mg/L) and luminol (1 mg/L) in 50 mM borate buffer (pH 9.3) (12). The flow rate of the CL reagent was 1.0 mL/min. The generated CL was monitored with a Chemiluminescence Analyzer OX-7 or CLD-100 (Tohoku Electronic Ind. Co., Sendai, Japan) equipped with a spiral teflon flow cell (cell vol., *ca.* 200 μL) (12–15). Chromatograms based on UV and CL detection were recorded simultaneously with a Sekonic SS-250F multiple recorder. All CL-HPLC assays were performed at 20°C in a temperature-controlled room.

Photooxidation of α-Toc. d-α-Toc (116 μmol; gift from Eisai Co., Tokyo, Japan) was dissolved in 25 mL of methanol containing 0.8 mg methylene blue (Wako Pure Chemical Co., Osaka, Japan) as a photosensitizer. The reaction mixture was placed in a beaker (200 mL) cooled with ice-cold water, and photoirradiated for 0 to 11 hr at 12.5°C. The light source, a 500-W photoreflector lamp (Toshiba Electronics Co., Tokyo, Japan), was held at a 50-cm distance above the surface of the reaction mixture. After photooxidation, the reaction mixture was passed through a silica gel column (20 × 150 mm, Wako Pure Chemical Co.) using methanol as eluant to also remove methylene blue. Toc and its oxidized products were recovered from the methanol extract under reduced pressure by rotary evaporation. The oxidation products of Toc were redissolved in 20 mL of methanol, and a 20-μL portion was subjected to CL-HPLC assay.

The hydroperoxide concentration was determined by KI reduction (16,17). The residual Toc in the photooxidized sample was measured by reversed phase HPLC using a Wakopak NU-ODS-10 column and fluorescence detection at Ex. 298 nm and Em. 325 nm (Jasco FP 210).

$NaBH_4$ reduction of photooxidized α-Toc. A 2-mL portion of the 10-hr photooxidized α-Toc was reacted in methanol with 100 μmol $NaBH_4$ (Wako Pure Chemical Co.) for 1 hr. The reaction products were recovered in the chloroform layer after adding chloroform and water to the

reaction mixture. The chloroform layer was dried, the residue redissolved in 2 mL of methanol, and 20-μL portions of the extract were subjected to CL-HPLC and α-Toc assay.

Thin-layer chromatography (TLC) of photooxidized α-Toc. The photooxidation products of α-Toc were applied to a TLC plate (DC-Kieselgel 60, Merck, Darmstadt, Germany) using hexane/diethyl ether (55:45, v/v) as the developing solvent. Spots were visualized either by charring with 50% sulfuric acid, or by spraying with 1% dimethyl-*p*-phenylenediamine/methanol solution. The hydroperoxide group containing compound gives a purple spot in TLC with the dimethyl-*p*-phenylenediamine reagent (18).

NMR and mass spectrometry. The chemiluminescent product that was revealed by CL-HPLC of photooxidized α-Toc was isolated by HPLC and characterized by ^{1}H NMR (Jeol JNM-GSX-270 NMR spectrometer) in $CDCl_3$ at 27°C. Isolated products were further analyzed by fast atom bombardment mass spectrometry (FAB-MS; Jeol JMN-HX-105 mass spectrometer). To avoid decomposition of the hydroperoxide of oxidized Toc, the chemiluminescent compound was collected by HPLC using a Jasco RPC-PO-based polymethacrylate column (4.6 × 150 mm, Japan Spectroscopic Co., Hachiohji, Tokyo, Japan) and methanol/water (95:5, v/v) as the mobile phase at a flow rate of 1.0 mL/min.

RESULTS

Figure 1 shows a typical chromatogram of 3-hr photooxidized α-Toc as obtained by CL-HPLC with OX-7 as the chemiluminescence detector. On the UV (240 nm) chromatogram, three major peaks were observed for oxidized Toc. The peak components were isolated by using normal-phase and reversed-phase HPLC, and were identified by ^{1}H NMR and mass spectrometry as being the same oxidation products as those found by Grams *et al.* (5) upon photoirradiation of α-Toc; that is, methoxy-tocopherone (8a-methoxy-α-tocopherone), epoxy-methoxy-tocopherone (mixture of *cis* or *trans*-4a,5-epoxy-*cis* or *trans*-8a-methoxy-α-tocopherone), α-Toc, and 8a-hydroperoxy-α-tocopherone (Toc-OOH). By comparison, on the CL chromatogram only a single intense peak was observed.[1] This single peak on the CL chromatogram was resolved under UV detection into an overlapping multiplet of 4 or 5 peaks. Rechromatography by CL-HPLC of an individual component that had been isolated from the multiplet fraction in UV-HPLC confirmed that only peak 1 is chemiluminescent (Fig. 1).

The intense CL peak completely disappeared when the photooxidized α-Toc was reacted with $NaBH_4$ (Fig. 2). Frei *et al.* (19) reported that chemiluminescence of ubiquinol did not disappear after reaction with $NaBH_4$ (19). This suggests that the CL peak observed in our experiment is not due to quinone derivatives such as Toc-quinone or Toc-quinone epoxide. When we examined authentic Toc-quinone by CL-HPLC, CL peaks due to Toc-quinone were not noted on the CL chromatograms (data not shown).

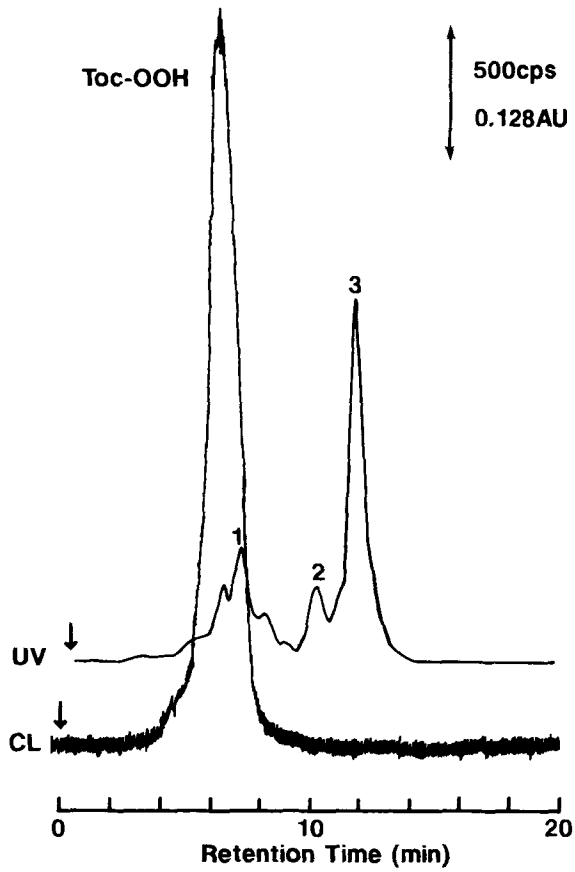

FIG. 1. Typical CL-HPLC chromatogram of the photooxidation products of α-Toc. Twenty-five mL of a methanolic solution containing 116 μmol *d*-α-Toc and 0.8 mg methylene blue were photoirradiated for 3 hr. The oxidation products were then treated as described in Materials and Methods and were injected into a CL-HPLC instrument equipped with an OX-7 detector. The main peaks identified were: 1, Toc-OOH (8a-hydroperoxy-α-tocopherone); 2, unoxidized α-Toc + 8a-methoxy-α-tocopherone; and 3, epoxy-8a-methoxy-α-tocopherone.

TLC of 2-hr photoirradiated α-Toc showed seven fractions (R_f 0.65, 0.63, 0.59, 0.49, 0.40, 0.37 and 0.22) upon charring with 50% sulfuric acid. α-Toc gave a spot at R_f 0.49 under the same conditions. By comparison, two spots (R_f 0.40 and 0.37) were identified as hydroperoxide-containing products as they gave positive results with the dimethyl-*p*-phenylenediamine reagent. These two fractions seemed to be the two epimeric products of 8a-hydroperoxy-tocopherone as previously reported by Yamauchi *et al.* (11,20). The structural characterization of the two fractions was further pursued by NMR and mass spectrometry.

The ^{1}H NMR spectrum of the chemiluminescent peak component isolated by HPLC clearly indicated the presence of a hydroperoxide group (Fig. 3). The proton signal due to the hydroxyl group (4.18 ppm) observed for α-Toc disappeared, and a doublet (7.34, 7.37 ppm) that can be ascribed to the hydroperoxide protons of the epimers was detected at lower field. The hydroperoxide signal disappeared after reduction of Toc-OOH with $NaBH_4$ (data

[1]When the new chemiluminescence detector CLD-100 was used, CL sensitivity improved; several small luminescent peaks ascribed as analogues of Toc-OOH appeared on the CL chromatogram (data not shown). However, these peaks showed little absorbance by UV detection, and were difficult to isolate and identify.

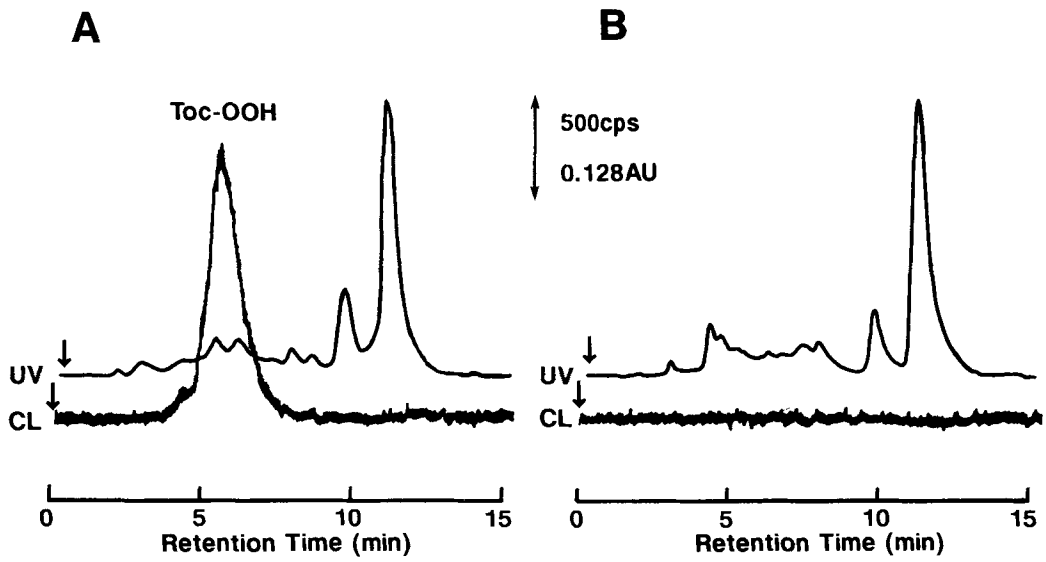

FIG. 2. Effect of $NaBH_4$ reduction of photooxidized α-Toc on CL-HPLC pattern. A, Photooxidized α-Toc; B, $NaBH_4$ reduction products of photooxidized α-Toc.

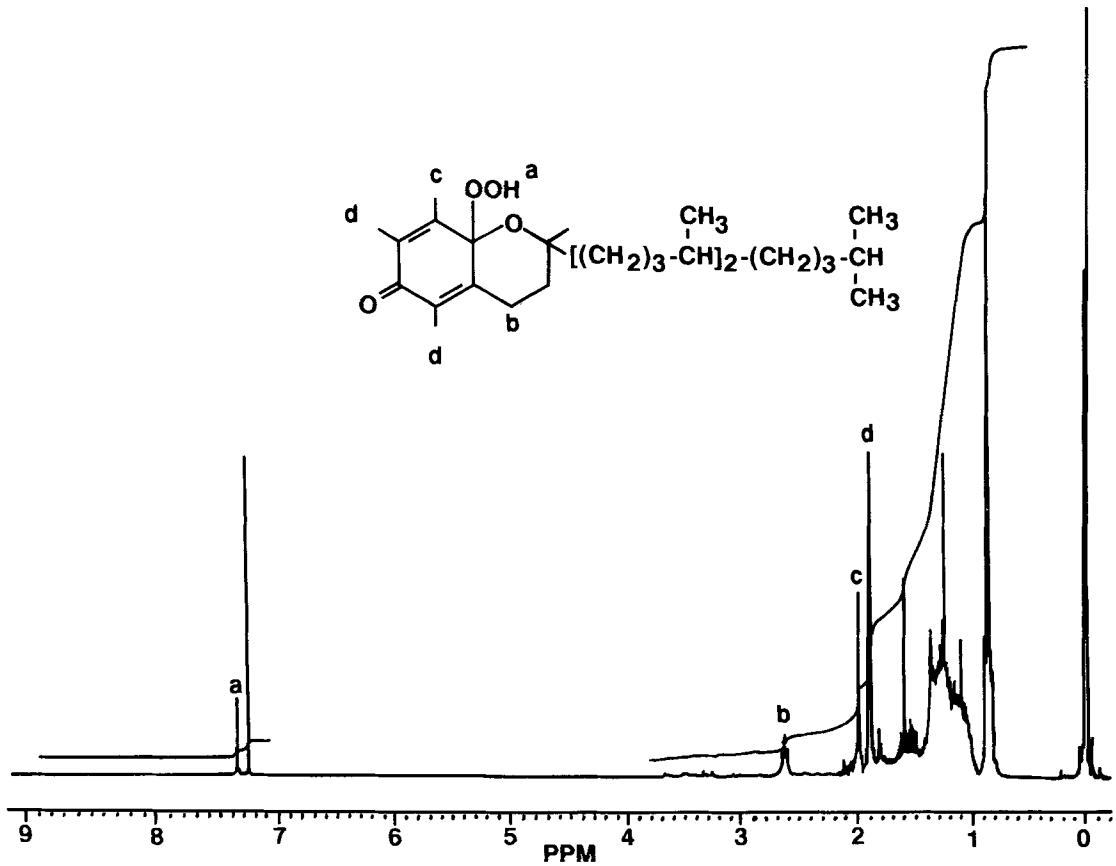

FIG. 3. ^1H NMR spectrum of the chemiluminescent product, 8a-hydroperoxy-α-tocopherone, from photooxidized α-Toc. The chemiluminescent product was isolated from photooxidized *d*-α-Toc prior to NMR analysis.

not shown). The methyl protons of the phytyl side chain appeared as a series of doublets at about 0.8 ppm (Fig. 3). The molecular weight of the chemiluminescent product was determined to be 462 by FAB-mass spectrometry, as expected for 8a-hydroperoxy-α-tocopherone. We therefore concluded that the chemiluminescent peak component, which we detected by CL-HPLC of the $^1O_2$/Toc oxidation products, was 8a-hydroperoxy-α-tocopherone.

Figure 4 shows the time-course of Toc-OOH formation, the changes in peroxide value (theoretical 4328 meq/kg for 8a-hydroperoxy-α-tocopherone), and the residual amount of α-Toc, when 116 μmol of α-Toc dissolved in methanol was subjected to photooxidation. After 30 min of photoirradiation, 90% of α-Toc remained, and just a small CL peak ascribed to Toc-OOH (corresponding to 9.1 μmol) was observed on the CL chromatogram. After 3-hr photooxidation, Toc had been completely oxidized. During this time, the increase in peroxide value of the oxidized Toc was proportional to the appearance of Toc-OOH in CL-HPLC. After 3-hr oxidation, 87.0 μmol of Toc-OOH (equivalent to 75.0% of initial α-Toc) was formed and the peroxide value of the oxidized Toc reached 3480 meq/kg. The decrease in Toc-OOH and peroxide value of oxidized Toc observed after the 3-hr photoirradiation is ascribed to the breakdown of Toc-OOH.

In order to improve sensitivity and to lower the detection limit of 8a-hydroperoxy-α-tocopherone, we also used the new chemiluminescence detector CLD-100, instead of OX-7. With the CLD-100, it was possible to extend the detection limit to as low as 50 pmol of 8a-hydroperoxy-α-tocopherone with our CL-HPLC system (Fig. 5). A mixture of cytochrome c (10 mg/L) and luminol (2 mg/L) in 50 mM borate buffer (pH 10.0) was favored as the CL reagent for measuring Toc-OOH. We found that at higher luminol concentration and pH, the sensitivity was generally increased, but under these conditions background was also increased. Therefore, the optimal concentration of luminol should be carefully chosen.

DISCUSSION

Toc is known to be the principal antioxidant in lipophilic biological systems and is thought to play an important role in protecting biomembranes from radical-induced injury. In rats, the molecular ratio of α-Toc to polyunsaturated fatty acids in liver and kidneys was reported to be below 0.001 (21). Tappel (22) suggested that ascorbic acid and/or reduced glutathione can synergistically regenerate Toc from Toc radicals. Similarly, Niki *et al.* (23) reported that in liposomal model systems ascorbic acid can regenerate Toc from its radical forms. Yamauchi *et al.* (20) suggested that 8a-hydroperoxy-tocopherone (Toc hydroperoxide) is convertible to Toc by ascorbic acid.

However, researchers have generally failed to detect Toc hydroperoxide among Toc oxidation products, because no selective and sensitive detection method had been applied until now. In this paper, we describe (for the first time) the hydroperoxide-specific detection of Toc-OOH with a CL-HPLC system and report that 8a-hydroperoxy-α-tocopherone can be identified as a prominent single peak in CL-HPLC.

Toc has been reported to inhibit chemiluminescence and has been shown to appear as a quenching peak in CL chromatograms (19). However, no quenching due to Toc (Rt, 9.8 min) was apparent on the chromatogram shown in Figure 1. This is because the base line was low under our conditions as methanol was used as HPLC solvent at a low luminol concentration. We should also emphasize

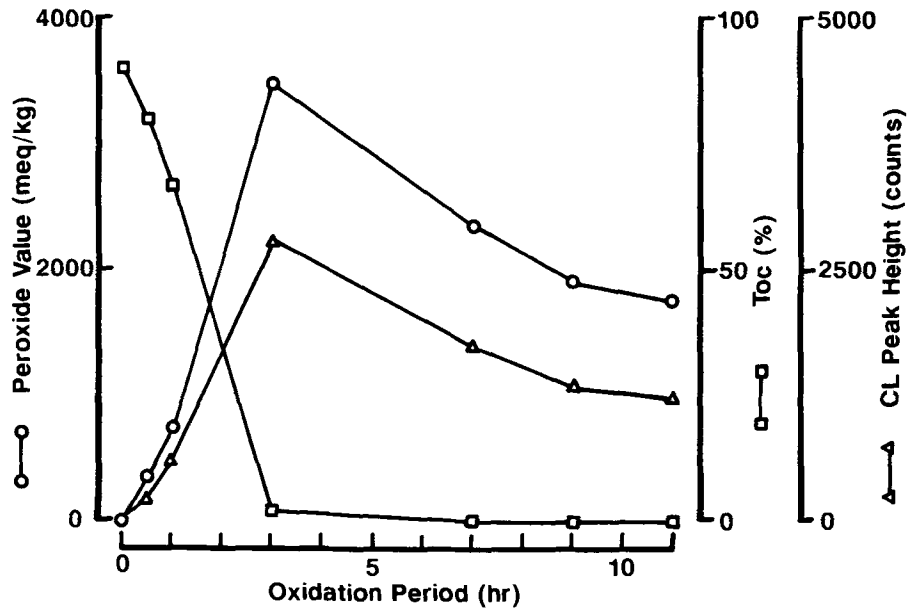

FIG. 4. Photooxidation time dependent changes of residual α-Toc, 8a-hydroperoxy-α-tocopherone (Toc-OOH) and peroxide value of oxidized α-Toc.

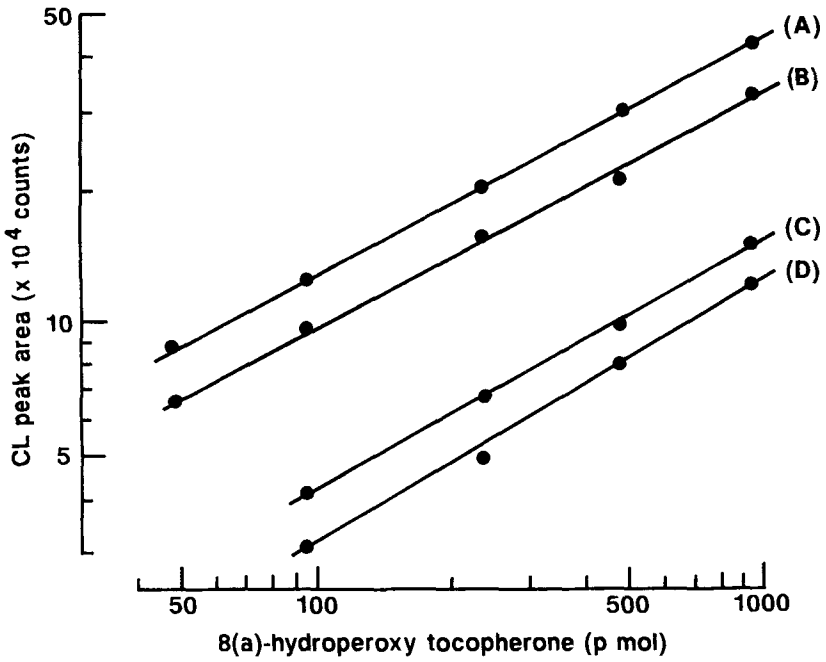

FIG. 5. Calibration curves of 8a-hydroperoxy-α-tocopherone. Photooxidized α-Toc was assayed by CL-HPLC using a CLD-100 analyzer and a RPC-PO polymethacrylate column. The mobile phase was methanol (flow rate, 1.1 mL/min). Four types of luminescent post column reagents (flow rate 1.0 mL/min) were tested: A, cytochrome c (10 mg/L) and luminol (2 mg/L) in 50 mM borate buffer (pH 10.0); B, cytochrome c (10 mg/L) and luminol (1 mg/L) in 50 mM borate buffer (pH 10.0); C, cytochrome c (10 mg/L) and luminol (2 mg/L) in 50 mM borate buffer (pH 9.3); and D, cytochrome c (10 mg/L) and luminol (1 mg/L) in 50 mM borate buffer (pH 9.3).

that α-Toc does not affect the chemiluminescence of 8a-hydroperoxy-α-tocopherone (Rt, 6.6 min) because of their different retention times.

Clough *et al.* (3) reported that 8a-hydroperoxy-α-tocopherone, which is a yellowish material, decomposed when passed through a silica gel column. This was confirmed in our experiments. For example, when we tested silica gel and ODS silica gel columns to isolate the peroxidized Toc with various solvent systems as eluants, we could not isolate the hydroperoxidic compound. Even in silica gel TLC at −25°C, it was impossible to isolate Toc-OOH. Despite decomposition of Toc-OOH during normal-phase silica gel column chromatography, in CL-HPLC chemiluminescent response was observed when a reversed-phase ODS column was used (Fig. 1). This implies that part of Toc-OOH is not destroyed and emits light, which allows the use of the ODS silica gel column for the detection of Toc oxidation products. Nevertheless, for complete protection of Toc-OOH, we prefer a polymer type column, such as the RPC-PO-based polymethacrylate column (Japan Spectroscopic Co., Tokyo, Japan). In the polymer column, Toc-OOH was more stable than in the silica gel column, and the detection limit was 50 pmol using the CLD-100 detector (Fig. 5).

We showed that the retention time of Toc-quinone was similar to that of Toc-OOH (Rt, 6.6 min) on the ODS silica gel column in CL-HPLC. However, Toc-quinone did not give a chemiluminescent peak. α-Toc quinone and α-tocopheryl-quinone-epoxide together with 8a-hydroperoxy-α-tocopherone have been reported to be formed by the reaction of α-Toc and photochemically generated $^1O_2$ (5–7). Grams *et al.* (5) reported that the major oxidation products were methoxy-tocopherone and epoxy-methoxy-tocopherone when Toc was reacted with $^1O_2$ in methanol. In our study, we detected methoxy-tocopherone and epoxy-methoxy-tocopherone together with Toc-OOH, and confirmed that only Toc-OOH is a chemiluminescent product (Fig. 1). It was thought that formation of methoxy adducts *via* Toc-OOH is a major oxidation pathway, while Toc quinone formation was a minor reaction when Toc oxidation with $^1O_2$ was performed in the methanol. Our results on methoxy adduct formation are, in principle, consistent with the findings of Grams *et al.* (5).

In the ^1H NMR spectrum that we measured at 27°C, the hydroperoxide protons of the epimers of 8a-hydroperoxy-α-tocopherone occurred at 7.34 and 7.37 ppm (Fig. 3), whereas those of the epimers at −25°C were reported at 8.9 ppm (3). As we proceeded to rather high temperatures, the hydroperoxide protons of Toc-OOH seemed to shift upfield, which may be the result of weakened hydrogen bonding with the oxygen at position-1 in the chroman ring.

Although oxidation processes of Toc with superoxide anion (24,25), singlet oxygen (20,26,27) and *t*-butyl hydroperoxide (28–30) have been studied extensively, detailed mechanisms have not yet been elucidated, especially for *in vivo* systems. Winterle *et al.* (31) reported that α-Toc formed 8a-alkylperoxy-α-tocopherones *via* reaction of alkylperoxyl radicals with α-tocopheroxyl radicals. Adduct

formation of peroxyl radicals and tocopheroxyl radicals was also reported by Liebler *et al.* (32) and Yamauchi *et al.* (11,20,33). These peroxyl radical dependent oxidation pathways of α-Tbc may also involve the formation of 8a-hydroperoxyl tocopherone as an intermediate which one should be able to detect by the present chemiluminescence assay. The CL-HPLC method should prove useful for the study of oxidoreductive reactions of tocopherols in foods and biological systems.

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An Extended Method for Separating and Quantitating Molecular Species of Phospholipids

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An improved and extended method for separating and quantitating molecular species of four phospholipid classes is presented. Crude lipid extract is first separated into phospholipid classes on a silica column. Each phospholipid class is then separated into molecular species without derivatization using high-performance liquid chromatography on columns packed with octadecyl silica. Quantitation of individual species is achieved by measuring absorbance at 205 nm. Factors for converting absorbancies to mol fractions have been determined. Quantitation by absorbance at 205 nm agrees well with quantitation by gas chromatography which is preferred to quantitation by phosphate analysis. One hundred phospholipid species have been identified. A table of relative retention times of molecular species is provided. Examples of quantitative analyses of species composition are presented. *Lipids 27,* **295–301 (1992).**

Fatty acids are attached to the 1- and 2-position of the glycerol moiety of phospholipids in a pattern that is charactistic of the particular class of phospholipid, of the source from which the lipid is derived, and of the dietary history of the organism. High-performance liquid chromatography (HPLC) has made possible the separation of each class of phospholipid into its molecular species, *i.e.,* the particular combination of fatty acids found in the 1- and 2-positions. Such separation can be accomplished on the intact phospholipid (1–3), or after it has been modified. In the latter case, the lipid is typically treated with bacterial phospholipase C to produce the corresponding mixture of diacylglycerol species which are then derivatized to form acetyl (4), benzoyl (5,6), 3,5-dinitrobenzoyl (7–9), or 1-anthroyl (10) derivatives. Trialkylsilyl derivatives of diacylglycerols also have been used (11,12). The amino groups of intact phosphatidylserine and phosphatidylethanolamine have been derivatized with 2,4,6-trinitrobenzenesulfonic acid or 1-fluoro-2,4-dinitrobenzene (13). These derivatives are separated into molecular species by HPLC. Earlier efforts to separate and quantitate molecular species have been reviewed by Smith and Jungalwala (1).

We report here an improved and extended method for separating and quantitating the molecular species of four phospholipid classes by high-performance liquid chromatography of the underivatized phospholipids. Quantitation is achieved by continuous absorbance measurement at 205 nm. Appropriate conversion factors have been determined for converting peak areas into mol fractions. The method of quantitation by absorbance agrees well with quantitation by gas-liquid chromatography (GLC). In cases where the HPLC resolution of molecular species is incomplete, the complex peak is analyzed and quantitated further by capillary GLC. The use of underivatized phospholipids makes it easier to preserve polyunsaturated fatty acids and avoids time-consuming enzymatic and chemical steps. For the present purpose, no attempt was made to separate alkenylacyl, alkylacyl and diacyl glycerophospholipids from each other prior to molecular species analysis.

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Abbreviations: BHA, butylated hydroxyanisole; CL, cardiolipin; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholine; NL, neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; RRT, relative retention time; SM, sphingomyelin.

MATERIALS AND METHODS

Materials. HPLC grade solvents and absolute ethanol were obtained from Fisher Scientific (Medford, MA) and U.S. Industrial Chemicals Co. (Tuscola, IL), respectively. Choline chloride was obtained from Sigma Chemical Co. (St. Louis, MO). Fatty acid methyl ester standards were purchased from Nu-Chek Prep, Inc. (Elysian, MN) and Applied Science (State College, PA). Ultrapure perchloric acid was purchased from Alfa Products (Danvers, MA).

Extraction of lipids. Total lipids from heart and liver of rats were extracted with chloroform/methanol (2:1, v/v) as described by Folch *et al.* (14). The tissue was weighed, homogenized in chloroform/methanol in an Omnimixer (Omni Corp. International, Waterbury, CT) and allowed to stand overnight under N_2 at room temperature before being filtered. C3H cells were washed, centrifuged at low speed, and extracted in similar fashion. The washed lipid extract was dried under a stream of N_2 and redissolved in 1 mL of isopropanol/hexane/ethanol (4.9:3.7:1.15, v/v/v) per g wet weight of tissue. The extract was stored under nitrogen at −20°C.

Separation of phospholipid classes. Column chromatography was performed on a Gradient Liquid Chromatograph (Model 334, Beckman Instruments, Fullerton, CA) equipped with an Variable Wavelength Spectrophotometer (Model 100-40, Hitachi Instruments, Elmsford, NY) and a Computing Integrator (Model 3390A, Hewlett Packard Co., San Fernando, CA).

Lipids were separated into phospholipid classes on columns packed with 10 μm particle-size LiChrosphere Si 100 (EM Science, Cherry Hill, NJ) by a modification of the method of Hax and van Kessel (15). Column dimensions were 4.6 × 250 mm for the small column (S) and 10 × 250 mm for the large column (L). The solvent mixtures used for eluting the lipids contained isopropanol/hexane/ethanol/1 mM ammonium phosphate/acetic acid in the proportions shown in Table 1. The solvent mixtures were filtered through a Millipore filter, type FH, pore size 0.5 μm (Millipore Corp., Bedford, MA), and then degassed. The column was equilibrated with 15 column bed volumes of Solvent A prior to use.

Lipid extract equivalent to 0.04–0.15 g wet weight of tissue was injected onto column S, which was then eluted according to the protocol shown in Table 1. The absorbance of the eluate was measured continuously at 205 nm and peak areas were integrated. Lipid extract equivalent to 0.2–0.5 g wet weight of tissue was injected onto column

TABLE 1

Elution Protocol for Class Separations

Time (min)	Solvents[a] A (%)	B (%)	Description
Column S ("Small", flow rate 1 mL/min)			
0-40	100	0	Isocratic
40-64	100-80	0-20	Linear gradient
64-104	80-0	20-100	Linear gradient
104-150	0	100	Isocratic
Column L ("Large", flow rate 3 mL/min)			
0-24	100	0	Isocratic
24-42	100-70	0-30	Linear gradient
42-66	70-50	30-50	Linear gradient
66-86	50-0	50-100	Linear gradient
86-120	0	100	Isocratic

[a]The solvents contained isopropanol/hexane/ethanol/1 mM ammonium phosphate/acetic acid in the following proportions, by volume: A, 490:370:115:25:0.4, and B, 490:370:60:80:0.4.

L, which was also eluted according to the protocol shown in Table 1. The absorbance of the eluate was measured continuously at 205 nm and peak areas were integrated. A typical elution pattern for lipids from rat heart is shown in Figure 1. Fractions of the eluate were collected and stored under Ar at −20°C. The 10 × 250 mm LiChrosphere Si 100 column exhibits virtually the same resolution at a flow rate of 6 mL/min, but the higher flow rate results in a shorter column life.

For consecutive runs, the LiChrosphere column was regenerated after each run with at least 10 column bed volumes of Solvent A, followed by two injections of 1.0 mL of absolute ethanol. The column was then washed with Solvent A until base line absorbance was achieved, before injecting the next sample. Before storing the column and

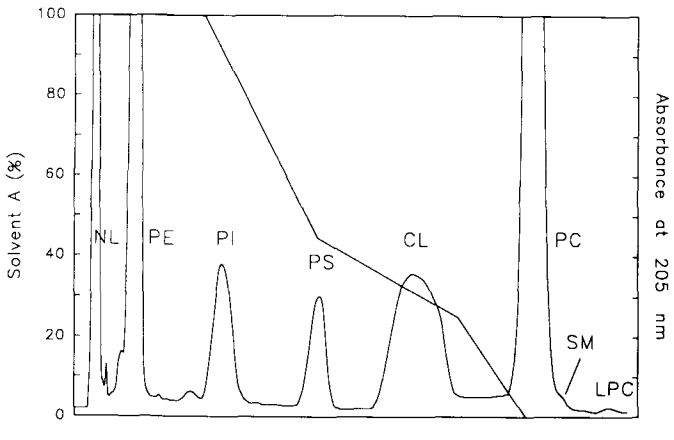

FIG. 1. Separation of phospholipid classes from rat heart. The column (10 × 250 mm) was packed with silica (LiChrospher Si 100). Extraction of lipids, application to the column, and elution conditions were as described under Materials and Methods. NL, neutral lipids; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine. For the compositions of Solvents A and B, see Materials and Methods. Full scale absorbance = 1.0.

before using it again, the column was washed with 15 column bed volumes of absolute ethanol.

If for any reason the column is not regenerated properly, phosphatidylserine (PS) may not separate completely from phosphatidylinositol (PI) during the class separation. This is readily detected during the molecular species separation of PS. The major PI species of 18:0-20:4ω6 then runs between the PS species 16:0-20:4ω6 and 18:0-20:5ω3, in a position not normally occupied by any PS species.

Sphingomyelin (SM) overlaps at the tail of the phosphatidylcholine (PC) peak (Fig. 1). If SM is a minor constituent, this is of little consequence in the molecular species analysis of phosphatidylcholine (PC). If SM is not a minor constituent, this is easily detected in the molecular species chromatogram of PC by the appearance of the 16:0 molecular species peak of SM (hereafter 16:0-SM). With 10 × log relative retention time (RRT) for the 16:0-22:6ω3 species of PC defined as 1.000, the 10 × log RRT for 16:0-SM is 0.930. Molecular species of PC to be expected in this region are shown in Table 2. In most analyses of mammalian phospholipids we have observed low amounts or no molecular species of PC in this region. An exception is 18:1-20:5ω3, which occurs in animals on a diet rich in eicosapentaenoate-containing fat or diets giving rise to 20:5ω3. Complete separation of PC from SM is achieved by re-running the PC/SM mixture on the same column (*i.e.*, LiChrosphere Si-100) using acetonitrile/methanol/water (80:12:8, v/v/v) as isocratic eluent. These proportions are similar to those used by Juaneda and Rocquelin (16).

Separation of molecular species. Fractions from the class separations which contain a particular phospholipid were pooled, dried under a stream of N_2, and redissolved in 0.5 to 2 mL methanol/water/acetonitrile (90.5:7.0:2.5, v/v/v) per g wet weight of liver. A portion of the resulting solution (100-500 μL) was injected onto a 4.6 × 250 mm column packed with octadecyl silica (5 μm particle size Ultrasphere ODS or Dynamax-300A; Rainin Instrument Co., Woburn, MA). For phosphatidylethanolamine (PE), PC and PI species, the column was eluted with methanol/286 mM aqueous choline chloride/acetonitrile (90.5:7.0:2.5, v/v/v) using a flow rate of 1 mL/min instead of the 2 mL/min recommended by Patton *et al.* (2). This change resulted in better resolution of the peaks. A flow rate of 0.5 mL/min gave an even better resolution. For PS species, the column was eluted with methanol/25 mM KH_2PO_4/acetonitrile/phosphoric acid (90.5:7.0:2.5:0.1, by volume), to which was added 0.42 g choline chloride per 100 mL. The mixture is similar to that used by Patton *et al.* (2), except that phosphoric acid is used in place of acetic acid. This change results in substantially better resolutions. Absorbance of the eluate was measured at 205 nm and recorded continuously. Fractions were collected and stored under N_2 at −20°C.

Gas-liquid chromatography. Fractions obtained during the species separations were dried under a stream of N_2 and transmethylated with 2% HCl in methanol, which was generated from acetyl chloride and "Lipopure" methanol (Alltech Associates, Deerfield, IL). The reagent (2-3 mL) was added to the dry sample, and the tube was sealed under nitrogen. The mixture was incubated at 70°C with occasional shaking for 60 min. The mixture was then diluted with 0.2*N* HCl and extracted four times with petroleum hydrocarbon. Alternatively, the transmethylation was carried out with 0.5*N* sodium methoxide in

TABLE 2

Relative Retention Times for Molecular Species of Phospholipids[a]

Peak no.	Log (10 × RRT)[b]	Species	Peak no.	Log (10 × RRT)	Species	Peak no.	Log (10 × RRT)	Species
1.	0.674	14:0-20:5ω3	34.	1.022	16:1ω7-22:4ω6	67.	1.200	18:1ω9p-22:5ω3
2.	0.722	16:1ω7-20:5ω3	35.	1.022	16:0-20:4ω6	68.	1.205	16:0-22:4ω6
3.	0.772	18:2ω6-20:5ω3	36.	1.027	18:1[c]-22:6ω3	69.	1.212	18:0p-20:5ω3
4.	0.772	14:0-22:6ω3	37.	1.050	16:0-18:2ω6	70.	1.214	18:1[c]-22:4ω6
5.	0.774	16:1ω7-16:1ω7	38.	1.052	18:1[c]-20:4ω6	71.	1.215	18:1[c]-18:1[c]
6.	0.796	14:0-20:4ω6	39.	1.058	17:0-16:1ω7	72.	1.226	18:0-22:6ω3
7.	0.800	15:0-20:5ω3	40.	1.068	16:0-22:5ω3	73.	1.235	16:0p-22:5ω6
8.	0.826	14:0-18:2ω6	41.	1.071	16:0-17:1ω7	74.	1.247	18:0-20:4ω6
9.	0.827	16:1ω7-22:6ω3	42.	1.072	15:0-18:1[c]	75.	1.262	16:0p-20:2ω6
10.	0.843	14:0-22:5ω3	43.	1.072	15:0-22:4ω6	76.	1.276	17:0-20:2ω6
11.	0.852	16:1ω7-20:4ω6	44.	1.080	18:1[c]-18:2ω6	77.	1.278	18:0-18:2ω6
12.	0.879	18:2ω6-22:6ω3	45.	1.092	16:0p-22:6ω3	78.	1.284	18:1ω9p-20:2ω6
13.	0.881	16:1ω7-18:2ω6	46.	1.096	18:1[c]-22:5ω3	79.	1.288	16:0p-18:1[c]
14.	0.886	15:0-22:6ω3	47.	1.098	18:1ω9p-22:6ω3	80.	1.293	16:0p-22:4ω6
15.	0.894	16:0-20:5ω3	48.	1.112	17:0-22:6ω3	81.	1.294	18:0-22:5ω3
16.	0.899	16:1ω7-22:5ω3	49.	1.114	16:0-20:3ω6	82.	1.311	18:1ω9p-18:1[c]
17.	0.902	18:2ω6-20:4ω6	50.	1.116	16:0p-20:4ω6	83.	1.315	18:1ω9p-22:4ω6
18.	0.922	18:1[c]-20:5ω3	51.	1.122	18:0-20:5ω3	84.	1.316	18:0p-22:6ω3
19.	0.931	14:0-20:2ω6	52.	1.131	16:0-22:5ω6	85.	1.317	17:0-22:4ω6
20.	0.932	18:2ω6-18:2ω6	53.	1.135	17:0-20:4ω6	86.	1.339	18:0p-20:4ω6
21.	0.940	15:0-18:2ω6	54.	1.140	18:1ω9p-20:4ω6	87.	1.340	18:0-20:3ω6
22.	0.944	16:1ω7-20:3ω6	55.	1.145	18:1[c]-20:3ω6	88.	1.348	19:0-20:4ω6
23.	0.950	18:2ω6-22:5ω3	56.	1.160	18:1[c]-22:5ω6	89.	1.355	18:0-22:5ω6
24.	0.956	16:0-16:1ω7	57.	1.160	16:0-20:3ω9	90.	1.363	18:0p-18:2ω6
25.	0.963	16:1ω7-22:5ω6	58.	1.166	17:0-18:2ω6	91.	1.384	18:0p-22:5ω3
26.	0.963	14:0-18:1[c]	59.	1.174	18:0-16:1[c]	92.	1.391	18:0-20:3ω9
27.	0.980	16:0-18:3ω6	60.	1.175	16:0-16:0	93.	1.396	16:0-20:1[c]
28.	0.987	16:0p[d]-20:5ω3	61.	1.178	16:0-20:2ω6	94.	1.403	18:0-20:2ω6
29.	0.992	18:2ω9-20:3ω6	62.	1.181	16:0p-22:5ω3	95.	1.412	18:0-18:1[c]
30.	0.994	16:1ω7-20:2ω6	63.	1.181	17:0-22:5ω3	96.	1.413	16:0p-22:2ω6
31.	1.000	16:0-22:6ω3	64.	1.185	18:1[c]-20:3ω9	97.	1.420	18:0p-20:3ω6
32.	1.021	16:1ω7-18:1[c]	65.	1.187	16:0-18:1[c]	98.	1.428	18:0-22:4ω6
33.	1.021	18:1ω9p-20:5ω3[c]	66.	1.198	16:0p-20:3ω6	99.	1.453	18:0p-22:5ω6
						100.	1.514	18:0p-18:1[c]

[a] The columns used were packed with octadecyl silica (Ultrasphere or Dynamax-300A; bed dimensions 4.6 mm diameter × 250 mm long; particle size 5 μm). The column was eluted with methanol/286 mM choline chloride/acetonitrile (90.5:7.0:2.5, v/v/v) at a flow rate of 1 mL/min. The data shown are average relative retention times of molecular species of phospholipids obtained from liver and heart of rats and from C3H cells grown in culture. The animals were kept on diets of laboratory chow ("Rat, mouse, hamster 3000," Agway Inc., Syracuse, NY), or diets containing 5% of either corn oil (Fleischmann's) or menhaden oil (MaxEpa, Schering) as the sole source of fat for two weeks. The cells were grown in Dulbecco's minimum essential medium supplemented with different long chain fatty acids for two weeks.

[b] RRT, relative retention time. The retention times are relative to the elution time of the species 16:0-22:6ω3.

[c] Mixed isomers of ω9 and ω7.

[d] Plasmalogens are indicated by "p".

methanol, freshly prepared with clean sodium metal and "Lipopure" methanol. The concentration was checked by titration with 0.2*N* HCl. The reagent (0.5 mL) was added to the dry sample and the tube was then sealed under nitrogen. The mixture was incubated at 50°C for 15 min. Acidification and extraction were done as before. The last step in preparing samples for GLC consists of drying the fatty acid methyl ester solution in petroleum hydrocarbon over anhydrous sodium sulfate. We have found that certain batches of anhydrous sodium sulfate obtained from different manufacturers can produce peaks in the gas chromatograms that are artifacts. A single recrystallization of the sodium sulfate from hot water, followed by drying in an oven at 150°C, was sufficient to remove the unidentified materials responsible for the artifacts. Fatty acid methyl esters were identified and quantitated by gas-liquid chromatography on a 50-m glass capillary column coated with Silar-5 CP, using methyl heptadecanoate as internal standard (17). Fatty aldehydes derived from plasmalogens were identified as dimethyl acetals. No attempt was made to identify alkyl ethers. The elution position of the 16:0-16:0 species, which has negligible absorbance at 205 nm, was determined and quantitated by GLC.

RESULTS

Typical elution patterns of species separations are shown in Figures 2–5. Zero time is the point at which the solvent front is observed by the detector. Relative retention times (RRT) of molecular species are shown in Table 2. RRT values were calculated relative to a retention time for 16:0-22:6ω3 of 1.000. In some instances it may be more convenient to use 18:0-20:4ω6 (10 × log RRT = 1.247; RRT = 1.766) as reference instead of 16:0-22:6ω3 (10 × log RRT = 1.000; RRT = 1.000, by definition). However, with the aid of Table 2, any identified species can be used as

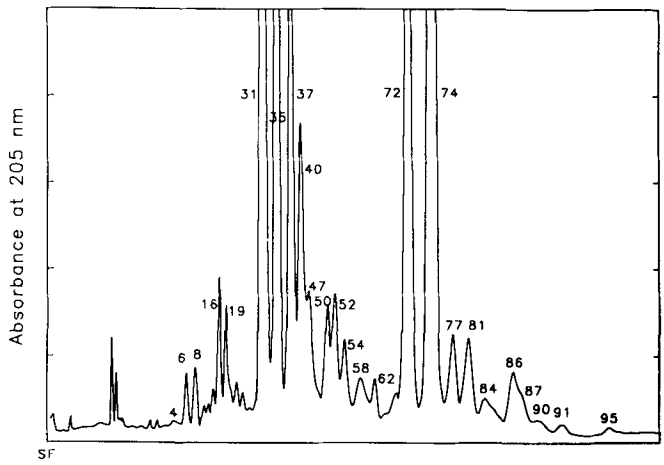

FIG. 2. Separation of molecular species of PE from rat liver. The column used was packed with octadecyl silica (Dynamax-300A). Elution conditions were as described under Materials and Methods, with a flow rate of 0.5 mL/min. Species are identified by the numbers assigned in Table 1. Full scale absorbance = 0.05.

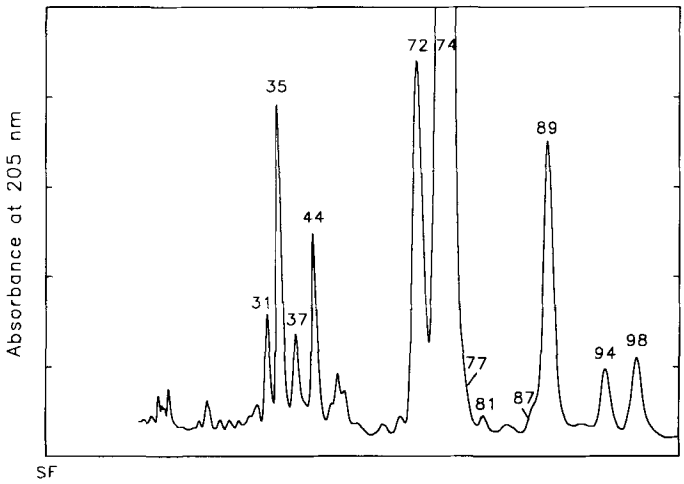

FIG. 4. Separation of molecular species of PS from rat liver. Elution and other details were as described in the legend to Figure 2, except that the flow rate was 1.0 mL/min.

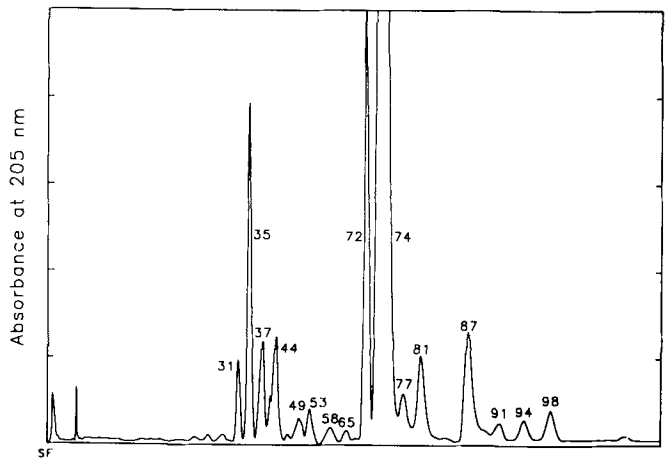

FIG. 3. Separation of molecular species of PI from rat liver. Elution and other details were as described in the legend to Figure 2.

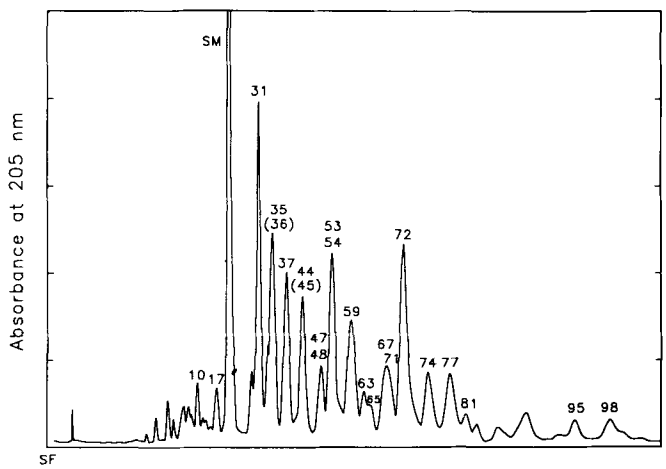

FIG. 5. Separation of molecular species of PC from rat liver. Elution and other details were as described in the legend to Figure 2, except that the flow rate was 1.0 mL/min. PC was used without separating it from SM, in order to illustrate the position of the 16:0-SM peak in the PC species chromatogram.

reference for identifying other species, or for confirming the identity of other species.

All species were identified initially by GLC after transesterification. Graphs of log (relative retention time of the species) *vs.* the equivalent chain length of the fatty acid found in the 1-position yielded a straight line for a given fatty acid in the 2-position (2). Repetition of this plotting method for different fatty acids in the 2-position yielded a series of parallel straight lines (Fig. 6). The equivalent chain length of saturated fatty acids is equal to the actual chain length. The equivalent chain length of unsaturated fatty acid and plasmalogens was determined as described in the legend to Figure 6. These graphs are used to identify molecular species in routine analyses. If conditions for regenerating and running the reverse phase HPLC column are kept constant, the relative retention times remain the same within ±2% and usually within ±1%. When changes in relative retention times do occur, the slopes of all lines change together in such a manner that they remain parallel. A slight change in the slopes may change the order in which peaks that run closely together are eluted. However, in practice we have found it unnecessary to construct new charts of relative retention times.

Conversion factors. The molecular species separated by reverse phase chromatography were quantitated by converting the areas of the ultraviolet absorbance peaks into chemical amounts. This necessitated the determination of conversion factors. The conversion factors for different peaks were obtained by dividing the area of the peaks by the chemical amounts in the peaks, which were determined by quantitative GLC using internal standards (17) or total phosphorus analysis. To carry out the latter, species peaks were taken to dryness, 0.4 mL $HClO_4$ was added, and the mixture was refluxed for 2–10 min depending on the amount of phospholipid. Orthophosphate was then measured by the method of Sanui (18). Table 3 shows

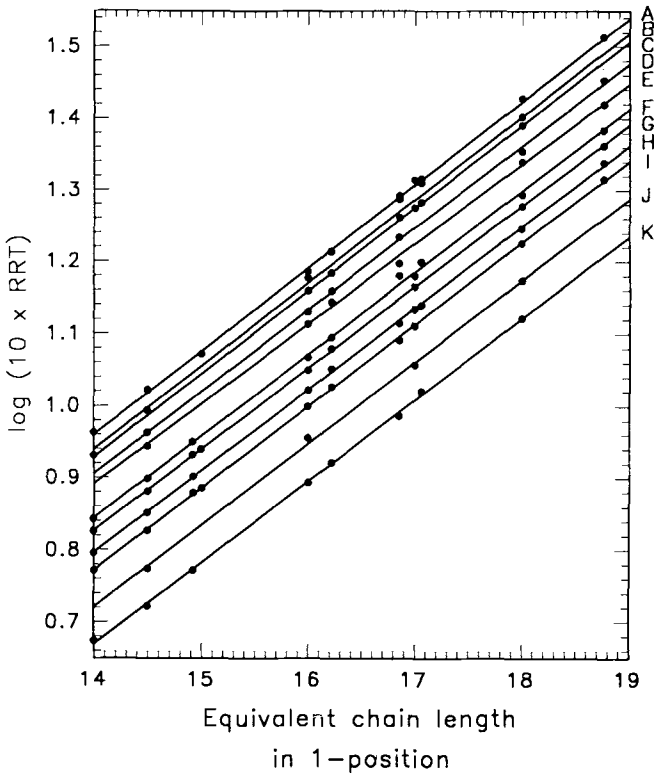

FIG. 6. Plot of log (10 × relative retention time) *vs.* equivalent chain length of fatty acids in 1-position of glycerophospholipids. The molecular species were identified as described in the text. Relative retention times for the same fatty acid in the 2-position were grouped together and plotted as a function the equivalent chain length in the 1-position. Linear regression lines are shown for each group. For saturated fatty acids in the 1-position, the equivalent chain length is equal to the actual chain length. For unsaturated fatty acids and alkenyl groups in the 1-position (plasmalogens) the equivalent chain length was determined to be as follows by drawing the best fitting vertical lines through the corresponding points on the individual regression lines: 16:1ω7, 14.50; 18:2ω6, 14.92; 18:1ω9&7, 16.22; 16:0p, 16.86; 18:1ω9p, 17.06; 18:0p, 18.77. Fatty acids in the 2-position were as follows: A, 18:1ω9&7 and 22:4ω6; B, 20:2ω6; C, 20:3ω9; D, 22:5ω6; E, 20:3ω6; F, 22:5ω3; G, 18:2ω6; H, 20:4ω6; I, 22:6ω3; J, 16:1ω7; and K, 20:5ω3.

conversion factors obtained with species containing 1,2, 4 and 6 double bonds. The conversion factors were plotted *vs.* the number of double bonds and the conversion factors for species with three and five double bonds were interpolated on the resulting graph. Table 3 also shows conversion factors that were determined for two species of plasmalogens. The conversion factor for 16:0-20:4 and 18:0-20:4 is 7.56. This yields a value of 7.56 ÷ 4 = 1.89 per double bond. The conversion factor for 18:0p-20:4 is 11.90, thus the additional vinyl ether double bond has a conversion factor of 11.90 − 7.56 = 4.34, and the vinyl ether linkage absorbs 4.34 ÷ 1.89 = 2.30 times more strongly than the aliphatic double bonds at 205 nm. The conversion factor for 16:0-22:6ω3 and 18:0-22:6ω3 is 10.4. This yields a value of 10.4 ÷ 6 = 1.73 per double bond. The conversion factor for 18:0p-22:6ω3 is 14.5; thus the additional vinyl ether double bond has a conversion factor of 14.5 − 10.4 = 4.1, and the vinyl ether linkage absorbs 4.1 ÷ 1.73 = 2.37 times more strongly than the aliphatic double bond at 205 nm. The two separate determinations agree within 3%. Gross (19) found that the vinyl ether bond absorbs 2.9 times more strongly at 203 nm than the aliphatic double bond. For molecular species containing two unsaturated fatty acids, the conversion factor was calculated by assuming that the conversion factors of the two fatty acids were additive. The conversion factors used by us have the dimension of 10^{-5} × area (in arbitrary units) per nmol phospholipid. The molecular species shown in Table 3 were quantitated by GLC. Two molecular species, 18:0-18:1 and 18:0-20:4ω6, were also quantitated by phosphorus analysis, with the same results.

The amount of each molecular species present is calculated as follows. The area of each peak is divided by the corresponding conversion factor to yield corrected areas (CA). The corrected areas are added to yield the total corrected area (TCA). The amount of each molecular species is then calculated in mol% using 100 × CA/TCA.

Depending on the composition of the lipid class, some molecular species eluted as overlapping peaks (Table 2 and Figs. 2-5). The fractions containing the overlapping peaks were analyzed by GLC. Methylheptadecanoate was used as internal standard to quantitate these species. The amount of each species so obtained was multiplied by its conversion factor, and then the areas were added to yield a calculated area for the peak in question. The calculated area agreed well with the integrated area of the peak, further validating this method of quantitation.

A template of a table which contains the molecular species shown in Table 2 has been created using the computer program Excel (Microsoft Corp., Redmont, WA). Quantitative molecular species analyses are recorded into this table. The template contains a series of equations, each of which calculates the total amount of a particular fatty acid in all molecular species. For example, all molecular species containing 16:0 are summed. This is repeated for each fatty acid and the total fatty acid composition in mol% is then calculated. The individual equations for each summation are stored in the table template and the calculations are made automatically. Lastly, the fatty acid composition in mol% obtained from the molecular species separation is compared with that obtained by GLC. When occasional discrepancies occurred between the two types of analyses during the development of the method, they were found to be due to misidentification of a molecular species in the HPLC chromatogram.

Representative results. Molecular species analyses of PS from livers of rats that were kept on two different diets are shown in Table 4. The method detailed here has been used to analyze the molecular species of PE, PI, PS and PC of C3H cells grown in media containing different fatty acid supplements (20).

DISCUSSION

The quantitative method described here does not require enzymatic or non-enzymatic degradation to diacylglycerols followed by derivatization. This presents a considerable saving in time and effort and eliminates errors due to incomplete degradation and derivatization as well as the potential for isomerization of diacylglycerol intermediates. The conversion factors used in calculating the actual amounts of each molecular species differ widely; however, the quantitative data obtained are highly reproducible and agree well with quantitative analyses

TABLE 3

Conversion Factors[a]

Double bonds per molecule	Species	Class	Conversion factors
			Measured values
1	16:0-18:1ω9 + ω7	PC	0.62 ± 0.07 (7)
2	18:0-18:2ω6	PC, PE	2.63 ± 0.19 (11)
4	16:0-20:4ω6	PC, PI	7.56 ± 0.27 (25)
4	18:0-20:4ω6	PC, PE, PI, PS	
6	16:0-22:6ω3	PC, PE	10.4 ± 0.8 (16)
6	18:0-22:6ω3	PC, PE	
			Interpolated values
3	16:0-20:3ω6		5.25
3	18:0-20:3ω6		
5	16:0-20:5ω3		9.32
5	18:0-20:5ω3		
5	16:0-22:5ω6 or ω3		
5	18:0-22:5ω6 or ω3		
			Measured values
5[b]	18:0p-20:4ω6	PE	11.9 ± 0.6 (3)
7[b]	18:0p-22:6ω3	PE	14.5 ± 0.4 (3)

[a]Areas of individual peaks were obtained by integrating the absorbance at 205 nm as a function of time. The area of each peak was then divided by the amount of phospholipid in the peak, which was determined by quantitative GLC. For further details, see Materials and Methods. Conversion factors are expressed as $10^{-5} \times$ area of peak (in integrator units) per nmol phospholipid ± standard deviation (number of determinations). The numerical values of these units depend on the integrator; however, the ratios of these values are constant and are used to calculate the mol% of each species.

[b]Including vinyl ether double bond.

TABLE 4

Molecular Species of Phosphatidylserine in Rat Liver[a]

	Species	Corn oil mol % ± S.D.	Fish oil mol % ± S.D.
Omega 3	18:0-20:5ω3		6.9 ± 0.2
	18:0-22:5ω3	0.17 ± 0.10	5.3 ± 0.1
	16:0-22:6ω3	0.50 ± 0.10	5.2 ± 0.1
	18:0-22:6ω3	4.7 ± 0.5	38.4 ± 2.8
	All other ω3	0.69	4.3
	Total ω3	6.1	60.1
Omega 6	18:0-18:2ω6	3.2 ± 0.3	0.28 ± 0.03
	18:0-20:2ω6	4.1 ± 1.6	
	18:0-20:3ω6	1.1 ± 0.16	0.93 ± 0.08
	16:0-20:4ω6	3.8 ± 0.3	0.52 ± 0.03
	18:0-20:4ω6	54.1 ± 2.2	6.0 ± 0.2
	18:0-22:4ω6	2.3 ± 0.7	
	18:0-22:5ω6	4.8 ± 0.5	0.63 ± 0.15
	All other ω6	4.3	2.1
	Total ω6	77.7	10.5
Omega 9&7	16:0-18:1ω9&7	1.7 ± 0.7	1.2 ± 0.2
	18:1ω9-18:1ω9&7	1.4 ± 0.7	
	18:0-18:1ω9	10.8 ± 3.4	19.6 ± 1.3
	18:0-18:1ω7	2.1 ± 0.7	8.0 ± 0.5
	All other ω9&7	0	0.55
	Total ω9&7	16.0	29.4
	Σω3/Σω6	0.077	5.73
	Σω6/Σω3	12.9	0.17

[a]Species are grouped in order of the position of the first double bond counted from the ω carbon atom of the fatty acid in the 2-position. Male rats were fed diets containing 0.025% BHA and 5% of either corn or fish oil (MaxEPA) as sole source of fat for two weeks. Results show mean ± S.D. for nine or five analyses, respectively. The animals were 53-days-old when analyzed. Only those species are shown which occurred in excess of 1 mol% in livers of animals on at least one of the two diets. A blank space indicates that the species was not detected in animals on one of the diets.

performed on the molecular species peaks. The separation of molecular species of phospholipids by HPLC also has been coupled to detection and identification by chemical ionization mass spectrometry (21).

The molecular species separations described in Figures 2–5 were run using octadecyl silica columns with an internal diameter of 4.6 mm. This required amounts of PE, PS and PI found in 50–250 mg of liver or heart. Preliminary work shows that the method can be adapted to octadecyl silica columns with an internal diameter of 1 mm with similar or better resolution. this makes possible the separation and quantitation of molecular species of phospholipids in 10–15 mg wet weight of tissue or cells.

No attempt was made to separate alkenylacyl, alkylacyl and diacyl glycerophospholipids from each other. The major species of alkenylacyl phospholipids were readily detected among the diacyl glycerophospholipids. Alkylacyl molecular species each account for a small fraction of the total molecular species (22). If necessary, alkenylacyl, alkylacyl and diacylglycerolipids can be separated from each other as described by Nakagawa and Horrocks (23). However, this involves removing the polar head group with bacterial phospholipase C, followed by acetylation, prior to molecular species analysis. The molecular species composition of cardiolipin (Fig. 1), which we did not investigate, has been studied by Teng and Smith (24) and Wolff *et al.* (25).

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COMMUNICATION

α-Helical Requirements for Free Apolipoproteins to Generate HDL and to Induce Cellular Lipid Efflux

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The structural requirement has been studied for apolipoproteins in their free form to interact with cells, to generate high density lipoprotein (HDL), and to cause cellular lipid efflux (*J. Biol. Chem. 266*, 3080-3086, 1991). It is shown that human apolipoprotein (apo) A-IV and apolipophorin III of *Manduca sexta* cause cholesterol efflux from cholesterol-loaded mouse peritoneal macrophages and reduce intracellularly accumulated cholesteryl ester as a result of forming HDL-like particles with cellular lipids, as do apoA-I, A-II and E. On the other hand, similar to apoC-III, reduced-and-carboxymethylated human apoA-II had no such effect. Thus, apolipoproteins seem to require at least four amphiphilic helical segments per molecule to express this function.
***Lipids 27*, 302-304 (1992).**

Removal of cholesterol from cells is the initial step of cholesterol transport from peripheral tissues to the liver, and high density lipoprotein (HDL) has been shown to function as an efficient acceptor of cellular cholesterol *in vitro* (1-5). When incubated with human plasma, cellular cholesterol taken up appears first in a minor HDL subfraction, "pre-βHDL", and then in other HDL fractions (6).

We have demonstrated that free apolipoproteins (apo) A-I, A-II and E in solution interact with macrophages and generate HDL-like particles with pre-β electrophoretic mobility, resulting in the release of cellular cholesterol and in lower levels of cellular cholesteryl ester (7). The K_m values of the reaction seem to be consistent with the putative free apolipoprotein concentrations in plasma or in peripheral lymph, since those values are as low as 0.25% of the apoA-I concentration in plasma (7-9). Thus, this reaction may play a physiological role in the first step of cholesterol removal from cells and appears consistent with the finding that cellular cholesterol appears first in "pre-βHDL" (6).

This reaction was observed with apoA-I, A-II and E while apoC-III did not show such an effect (7) although the latter protein binds to lipids as strongly as do the three former apolipoproteins (10-12). It appears that some parameter related to apolipoprotein chain length, such as the number of amphiphilic helical segments (11,13-15), may be one of the determining factors for formation of HDL-like particles rather than the presence of specific domains that would be required for the interaction. In order to test this hypothesis, complementary experiments were carried out with a series of apolipoproteins, including human apoA-IV, reduced-and-carboxymethylated human apoA-II (CMapoA-II) (16), and insect apolipophorin III (apoLpIII) from *Manduca sexta* (17).

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Abbreviations: apo, apolipoprotein; apoLpIII, apolipophorin III; CM, reduced and carboxymethylated; HDL, high density lipoproteins; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate.

MATERIALS AND METHODS

ApoA-I, A-II and apoE were isolated from human plasma as previously described (10,12). ApoA-IV was isolated from fresh human plasma according to the method described by Weinberg and Scanu (18). The bottom fraction of the density 1.21 g/mL adjusted with NaBr was dialyzed against 5 mM Na-phosphate buffer, pH 7.4, containing 0.15 M NaCl, and then diluted with the same volume of the buffer, to which NaCl was added at 0.28 g/mL. The fraction was then mixed with 20% Intralipid (KabiVitrum Canada, Newmarket, Ontario) at a ratio of 3:1 (v/v) and incubated at 37°C for 1 hr. The lipid emulsion was isolated by centrifugation and delipidated with ethanol/diethyl ether (1:3, v/v). Protein was applied to a DEAE/cellulose column using a gradient from 50 to 90 mM Tris-HCl, pH 8.2, containing 8 M urea. The fraction containing apoA-IV was rechromatographed under the same condition to obtain pure apoA-IV, which was identified as a single band with a molecular weight of 45,000 in polyacrylamide gel electrophoresis in the presence of 1% sodium dodecylsulfate (SDS-PAGE). ApoLpIII was isolated from hemolymph of *Manduca sexta* (17) and kindly provided by Dr. Robert Ryan, Lipid and Lipoprotein Research Group, University of Alberta, Edmonton, Alberta, Canada. The protein migrated as a single band in SDS-PAGE. ApoA-II was reduced and carboxymethylated by iodine acetate in the presence of β-mercaptoethanol as previously described (16,19). The modified apoA-II migrated as a single band with an apparent molecular weight of 8700 in SDS-PAGE. All apolipoproteins were lyophilized and stored under argon at −75°C. Aqueous solutions of each protein were prepared in a manner as described previously (10, 12,17). Protein concentrations of the solution were determined by absorbance at 280 nm using the specific molar extinction coefficient for each protein.

Low density lipoprotein (LDL) was labeled with [1,2-^{3}H]-cholesteryl oleate (45.4 Ci/mmol, Amersham, Arlington Heights, IL) as described (7,20) and then acetylated (7,21). Mouse peritoneal macrophages were obtained from ICR mice by peritoneal lavage, and approximately 2×10^6 cells were placed in each dish (3.5 cm). Cells adhering to the dishes were incubated with labeled acetylated LDL for 24 hr (and with the [*methyl*-^{3}H]choline chloride, Amersham, when phospholipids were also labeled), and then for another 24 hr without lipoprotein at 37°C (7). The cells were then exposed to the apolipoprotein solutions in the medium at 37°C for a certain period of time (7). The lipids were extracted from the medium and the cells, analyzed by thin-layer chromatography, and radioactivity in free and esterified cholesterol (and in each choline-containing phospholipid when labeled) was determined as previously described (7). Specific radioactivities of free and esterified cholesterol were determined for a few experimental points in each series of the experiment, based on quantitation of the lipids by gas chromatography. An average value was used for calculation of cholesterol levels of

other experimental points based on radioactivity (7). To determine the density of the peak for cholesterol, the medium was analyzed by sucrose density gradient ultracentrifugation as previously described (7).

RESULTS AND DISCUSSION

Figure 1 shows the change in cellular cholesterol and its efflux into the medium resulting from incubation of the cells with apoLpIII and apoE. The release of free cholesterol from cholesterol-loaded macrophages into the medium was demonstrated with both apolipoproteins. The reciprocal decrease in intracellular cholesterol was demonstrated for esterified cholesterol. In both cases, the rates were dose-dependent showing a saturation kinetics profile. K_m values obtained from double reciprocal plots were 1-1.5 $\times$ 10^{-6} M and 4 $\times$ 10^{-7} M for apoLpIII and apoE, respectively. The results were highly consistent with those previously reported for apoA-I, A-II and E (7). Figure 2 shows the release of free cholesterol into the medium as mediated by apoA-II, CMapoA-II, and by apoA-IV. Substantial efflux of free cholesterol was demonstrated with apoA-II consistent with previous work (7), but CMapoA-II did not cause cholesterol release from the cells. On the other hand, apoA-IV was shown to be capable of removing cellular cholesterol. Analysis of the medium in case of apoLpIII by density gradient ultracentrifugation showed peaks for free cholesterol, phosphatidylcholine and sphingomyelin all at the density of 1.1 g/mL (Fig. 3), consistent with the results previously obtained with apoA-I, A-II and E (7).

All these apolipoproteins contain amphiphilic α-helical segments of some 22-24 amino acid residues which are thought to be responsible for interactions with lipids (11, 13-16,22,23). Table 1 lists the apolipoproteins examined

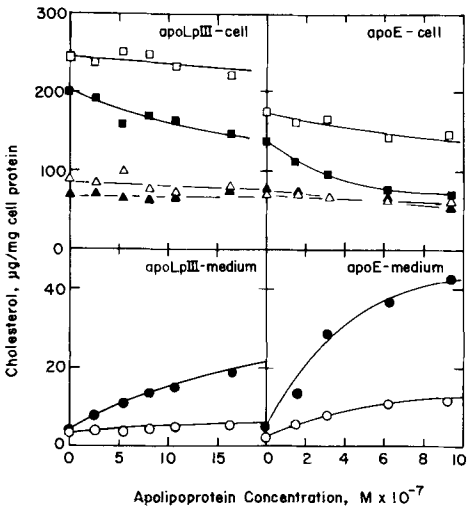

FIG. 1. Release of free cholesterol from macrophages and reciprocal decrease in intracellular cholesterol induced by apolipoproteins. Cholesterol-loaded macrophages were exposed to a solution of human apoE or apoLpIII of *Manduca sexta* for 6 hr (open symbols) or 24 hr (closed symbols). Free cholesterol in the medium (circles), and free and esterified cholesterol in the cells (triangles and squares, respectively) were determined as described in the Methods section (8). Cell protein per dish was 43.8 $\pm$ 5.0 μg for apoLpIII and 81.8 $\pm$ 10.3 μg for apoE.

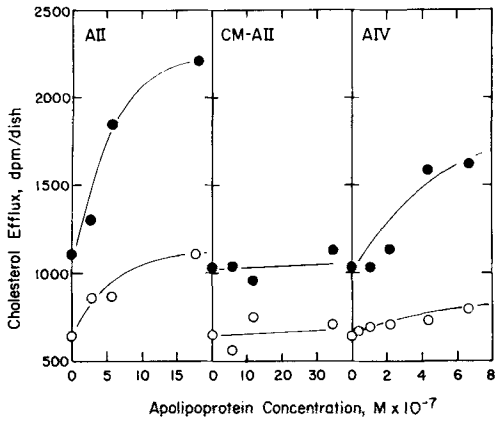

FIG. 2. Apolipoprotein-induced release of cholesterol from macrophages into the medium. ApoA-II, left; CMapoA-II, middle; apoA-IV, right. Cholesterol-loaded macrophages were incubated with apolipoproteins for 6 hr (open circles) or 24 hr (closed circles). Specific radioactivity of cholesterol is approximately 4000 dpm per μg of total cellular cholesterol. Cell protein per dish was 25.6 $\pm$ 2.9 μg.

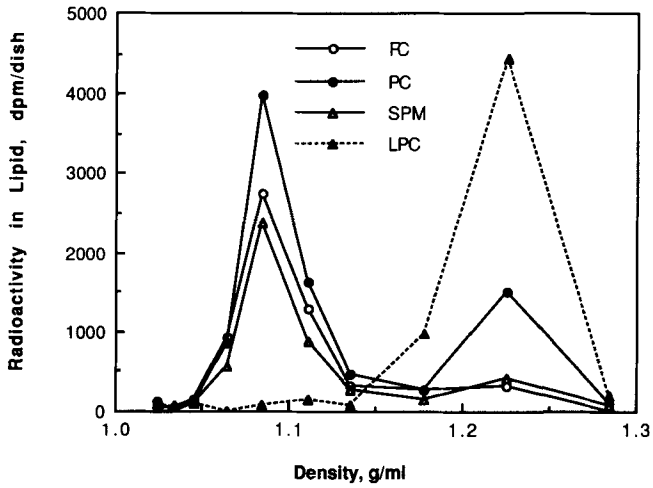

FIG. 3. Density gradient ultracentrifugation of the medium containing apoLpIII after incubation with macrophages labeled with [^{3}H]cholesterol and [^{3}H]choline. Cell protein per dish was 15.3 $\pm$ 1.1 μg. Symbols: free cholesterol, (○); phosphatidylcholine, (●); sphingomyelin, (△); lysophosphatidylcholine (▲).

TABLE 1

Cellular Cholesterol Efflux Induced by Free Apolipoproteins and Their Number of Amphiphilic Helices

Apolipoprotein	Number of amino acid residues	Number of helices[a] (reference)	Cholesterol release from macrophages[b]
ApoA-IV	376	≈ 12 (22)	+
ApoE	299	8 (15)	+
ApoA-I	243	6 (14)	+
ApoLpIII	166	5 (23)	+
ApoA-II	77 × 2	≈ 4 (11)	+
CMapoA-II	77	≈ 2	−
ApoC-III	79	≈ 2 (13)	−

[a] Number of amphiphilic helical segments of 20 to 24 amino acids per molecule as predicted (references).

[b] Capability of apolipoproteins to generate HDL and to cause cellular lipids efflux upon interaction with cholesterol-loaded macrophages.

and gives the putative number of such segments in each molecule. The data suggest that apolipoproteins with more than four such segments are capable of removing cellular cholesterol by generating HDL-like particles, whereas those with two or less such segments can not.

ApoC-III and CMapoA-II both contain at most only two amphiphilic helical segments. ApoC-III is in a random structure and does not self-associate in aqueous solution (10,12,24). However, it binds to the lipid surface as strongly as do other apolipoproteins whereby an amphiphilic helical structure is induced (10–12). Human apoA-II is a disulfide-linked homodimer and its carboxymethylation generates two identical monomers which differ from the original monomer only by the modified cystein at residue 6. CMapoA-II strongly self-associates in aqueous solution, thereby inducing a helix, and binds to the lipid surface (16). However, neither generate HDL-like particles upon interaction with macrophages.

Most vertebrate apolipoproteins except apoB, including those examined in this study, are likely to belong to a gene family under the same evolutionary tree (25,26) having common structure of multi-segments of amphiphilic helices. However, sequence homology is not strikingly high among them, so that it seems difficult to postulate that these apolipoproteins interact with the cells *via* a specific domain. ApoA-I, A-II and A-IV complexed with phospholipid reportedly bind to the same membrane "receptor site" and promote cholesterol efflux from adipocytes (27). This may argue against specific interactions between these apolipoproteins and membrane protein(s). Furthermore, cholesterol removal has been demonstrated with insect apoLpIII from *Manduca sexta*, which is poorly homologous to human apolipoproteins in terms of amino acid sequence (28), but is similar in function to human apolipoproteins in regard to physicochemical properties (28,29) based on its amphiphilic helical segments (23). It is therefore unlikely that any specific domain, such as a recognition site for a cell membrane protein, could be required for interaction of free apolipoproteins with cholesterol-loaded macrophages to generate HDL-like lipoproteins under removal of cellular cholesterol.

Thus, it seems that lipid-protein interactions mediated by amphiphilic helices are mainly responsible for generation of HDL upon interaction with the cell surface. However, not all the parameters for lipid-protein interaction of apolipoproteins directly correlate with this. ApoA-I, A-II, A-IV and E spontaneously generate disc-like particles with certain phospholipids such as dimyristoyl phosphatidylcholine (27,30–32), but CMapoA-II does not form typical "discs" even with this phospholipid (33). However, apoC-III does form the disc-like particles with dimyristoyl phosphatidylcholine although it has a peptide chain of about the same length as dose CMapoA-II, and both are incapable of causing cellular lipid efflux (34). The apparent dissociation constants of apoA-I, A-II, C-II and C-III are all in the same order when they are measured for the surface of phospholipid/triglyceride microemulsion having a diameter of 26 nm (10), while the dissociation constant of apoE is significantly higher (12). Therefore, we cannot completely rule out a more specific interaction between the membrane and certain apolipoproteins as a contributing mechanism for the generation of HDL.

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Inhibition of Myocardial Lipoprotein Lipase by U-57,908 (RHC 80267)

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U-57,908 (RHC 80267) was shown to inhibit lipoprotein lipase (LPL) activity in cardiac myocytes from rat hearts; the concentrations required for inhibition to 50% of control activity were 1.1 μM and 2.5 μM for myocyte homogenates and a post-heparin medium preparation, respectively. The inhibition of LPL activity by U-57,908 was not changed when the concentration of the triolein substrate and apolipoprotein CII activator in the assay was reduced. The availability of U-57,908 as a potent and selective LPL inhibitor may provide a useful experimental approach in studies on lipoprotein metabolism.
Lipids 27, **305–307 (1992).**

Lipoprotein lipase (LPL) catalyzes the hydrolysis of the triacylglycerol component of circulating lipoproteins (1). A selective inhibitor of LPL activity that could be utilized with either intact cell and organ preparations or under *in vitro* conditions would be useful. Antibodies to LPL have been utilized to inhibit LPL activity bound to the surface of the capillary endothelium in perfused hearts (2) or to cultured vascular endothelial cells (3), but inhibitory antibodies are not generally available for experimental use.

U-57,908, *i.e.* cyclohexanone-*O,O'*[(1,6-hexanediyl)-*bis*-(iminocarbonyl)]dioxime, which was formerly designated RHC 80267 (4), has been characterized as a potent inhibitor of diacylglycerol (DAG) lipase activity in extracts of platelets (4), pancreatic islets (5), cardiac myocytes (6) and gastric smooth muscle (7). In contrast, the activities of phospholipase A_2 (4,5,7), phospholipase C (4,5) and DAG kinase (6,7) are not reduced by U-57,908 (RHC 80267). Consequently, U-57,908 has been utilized to determine whether the degradation of DAG, derived from phospholipids, by a DAG lipase pathway (8) could release arachidonic acid. The supply of free arachidonic acid regulates a variety of biological processes such as hormone secretion (5,9) and prostanoid biosynthesis (7). In an investigation of the ability of U-57,908 to inhibit DAG metabolism in isolated cardiac myocytes, U-57,908 was observed to inhibit LPL activity in myocyte homogenates (6). Therefore, our objective in this investigation was to further characterize the inhibition of LPL activity from cardiac myocytes by U-57,908.

MATERIALS AND METHODS

Preparation of cardiac myocytes. Calcium-tolerant cardiac myocytes were isolated from the hearts of male Sprague-Dawley rats (200–300 g), essentially as described by Kryski *et al.* (10). The freshly isolated myocytes were resuspended in Joklik minimal essential medium supplemented with 1.2 mM $MgSO_4$, 1 mM carnitine, 1.5 mM $CaCl_2$ and 1% (w/v) defatted albumin (10) to a cell density of 4×10^5 cells/mL. A cell pellet was collected by centrifugation and sonicated (6×30 s, using a BraunSonic (B. Braun Instruments, San Francisco, CA) 1510 sonicator at 75 W) into 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM *N*-(2-(hydroxyethyl)piperazine-*N'*(2-ethanesulfonic acid) (HEPES), pH 7.4, to give a homogenate for the assay of cellular LPL activity. In some experiments, myocytes (4×10^5 cells/mL of Joklik medium) were incubated for 30 min with 5 U/mL heparin (11) to displace LPL bound to the cell surface; after centrifugation, the supernatant (post-heparin medium) was collected for LPL assays.

LPL assay. The activity of LPL in myocyte homogenates (sonicated cells) and in the post-heparin incubation medium was assayed with a sonicated [^{3}H]triolein (glycerol-[9,10-^{3}H]trioleate) substrate emulsion (12). The standard assay conditions were: 0.6 mM [^{3}H]triolein (1 mCi/mmol), 25 mM piperazine-*N,N'-bis*(2-ethanesulfonic acid) (PIPES), pH 7.5, 0.05% (w/v) defatted albumin, 50 mM $MgCl_2$, 1 μg/mL apolipoprotein CII as the LPL activator, and either 20 μL homogenate or 100 μL of post-heparin medium. After a 30-min incubation at 30°C, the release of [^{3}H]oleic acid was determined by liquid-liquid partitioning (12). All assays were performed in duplicate; LPL activity was linear with respect to incubation time (10–40 min) and the content of myocyte sonicates or post-heparin medium, and is routinely expressed as nmol oleate released per hour per 10^6 cells. Lipase activity in myocyte homogenates determined in the absence of apolipoprotein CII and in the presence of anti-LPL antibody is less than 10% of apolipoprotein CII-stimulated (LPL) activity (13). Therefore, other tissue lipases do not contribute significantly to LPL assays with sonicated cardiac myocytes.

Materials. [^{3}H]Triolein was purchased from Amersham Canada (Oakville, Ontario, Canada). Human apolipoprotein CII and U-57,908 were generous gifts from Dr. P. Connelly (University of Toronto, Toronto, Ontario, Canada) and Dr. D. R. Morton (The Upjohn Company, Kalamazoo, MI), respectively. A 1-mM stock solution of U-57,908 was prepared in dimethylsulfoxide; control assays contained appropriate quantities of solvent which had no effect on LPL activity.

RESULTS AND DISCUSSION

U-57,908 inhibited LPL activity concentration-dependently in myocyte homogenates (Fig. 1), confirming previous results from this laboratory (6). LPL activity was reduced to less than 5% of control at 10 μM U-57,908; a concentration of 1.1 μM resulted in a 50% inhibition of control activity. LPL in myocyte homogenates has been reported to be predominantly particulate after differential centrifugation (13).

LPL can be released from surface binding sites on cardiac myocytes into the medium following incubation with heparin (11,14). Soluble LPL in the post-heparin medium

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Abbreviations: DAG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*(2-ethanesulfonic acid); LPL, lipoprotein lipase; PIPES, piperazine-*N,N'-bis*(2-ethanesulfonic acid); U-57,908, cyclohexanone-*O,O'*[(1,6-hexanediyl)-*bis*(iminocarbonyl)]dioxime.

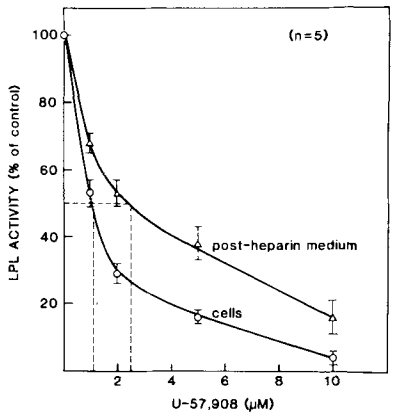

FIG. 1. Concentration-dependent inhibition of LPL activity by U-57,908. LPL activity in myocyte homogenates (○) or post-heparin medium (△) was determined in the presence of the indicated concentrations of U-57,908. Results are the mean ± S.E. ($n = 5$) and are expressed as the percentage of control activity determined in the absence of U-57,908. Absolute values of LPL activity corresponding to 100% control were 1190 and 156 nmol/hr/10^6 cells for myocyte homogenates and post-heparin medium, respectively.

was less sensitive to inhibition by U-57,908 (inhibition to 50% of control at 2.5 μM U-57,908) than the enzyme in cell sonicates (Fig. 1). Nevertheless, both preparations of LPL from cardiac myocytes were more sensitive to inhibition by U-57,908 than DAG lipase. The activity of DAG lipase in a variety of preparations was inhibited to 50% of control activity at U-57,908 concentrations ranging from 3.5–6 μM (4–6).

The inhibition of myocardial LPL activity by U-57,908 was constant when the time of incubation was varied from 10–40 min, so that the assay time-course was still linear in the presence of the inhibitor. Preincubation of post-heparin medium with 5 μM U-57,908 followed by a five-fold dilution into the assay indicated that the inhibition was reversible.

The inhibition of LPL in either myocyte homogenates or post-heparin medium by 2 μM U-57,908 was not changed when the triolein substrate concentration was reduced from 0.6 mM (standard assay conditions) to 0.1 mM (Table 1), suggesting that U-57,908 is not likely to be a competitive inhibitor of LPL activity with respect to substrate. The inhibition of DAG lipase activity in a microsomal subcellular fraction from cardiac myocytes by U-57,908 was also reported to be non-competitive with respect to dioctanoylglycerol as substrate (6).

The inhibitory effect of U-57,908 on LPL activity was also unchanged when assays were performed with a sub-optimal concentration (0.1 μg/mL) of apolipoprotein CII as the LPL activator (Fig. 2). Similar results were obtained when two concentrations of chicken serum (0.5% and 2%, v/v) replaced apolipoprotein CII in the LPL assay (results not shown).

In summary, U-57,908 was a potent and effective inhibitor of LPL activity, as assessed under a variety of *in vitro* assay conditions (varying concentrations of triolein substrate and apolipoprotein CII activator) with two different enzyme preparations (cell homogenates and post-heparin medium) from cardiac myocytes. LPL catalyzes the release of fatty acids from the *sn*-1 and *sn*-3 positions of triacylglycerols (15). Therefore, U-57,908 appears to be a relatively selective inhibitor for the release of fatty acids from the primary positions of acylglycerol substrates, since U-57,908 was a potent inhibitor of the hydrolysis of the *sn*-1 position of a 1-palmitoyl-2-oleoyl-*sn*-glycerol substrate (DAG lipase activity) but was much less effective as an inhibitor of 2-monoacylglycerol lipase activity in cardiac myocytes (6). Rindlisbacher *et al.* (16) have also reported that U-57,908 (RHC 80267) inhibited DAG lipase activity (*sn*-1 hydrolysis) but not 2-monoacylglycerol lipase activity in plasma membranes from adrenal chromaffin cells. LPL is bound to the luminal surface of capillary endothelial cells where the enzyme has its functional role in lipoprotein metabolism (1). U-57,908 may provide a particularly useful experimental tool as an LPL inhibitor in

TABLE 1

Effect of Triolein Substrate Concentration on the Inhibition of LPL Activity by U-57,908

Preparation	U-57,908 (μM)	LPL activity[a] (nmol/hr/10^6 cells) 0.6 mM Triolein	0.1 mM Triolein
Cell sonicate	0	1330	569
	2	294 (22%)[b]	138 (24%)
Post-heparin medium	0	156	60
	2	70 (45%)	28 (46%)

[a] Results are the mean from 2 experiments.

[b] Percentage of control activity measured in the absence of U-57,908.

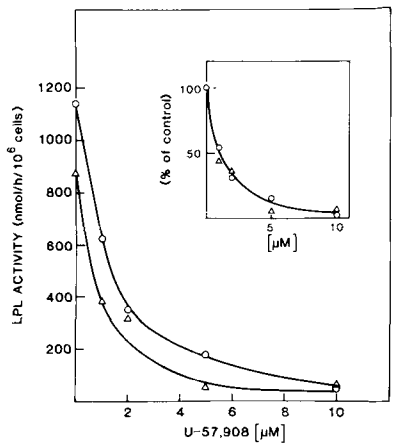

FIG. 2. Effect of apolipoprotein CII on the inhibition of LPL activity by U-57,908. LPL activity (nmol/hr/10^6 cells) in a myocyte homogenate was determined in the presence of either 0.1 μg/mL (△) or 1 μg/mL apolipoprotein CII (○) and the indicated concentrations of U-57,908. Insert: LPL activity, expressed as a percentage of the respective control, is replotted as a function of the concentration of U-57,908. Similar results were obtained in a second experiment.

perfused organs and tissues, because cellular uptake of the inhibitor will not be necessary, and therefore, any potential effect of concurrent inhibition of intracellular DAG lipase on cellular function will be minimized. The demonstration that U-57,908 can be an effective LPL inhibitor *in vivo* is an important future research objective.

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Glycolipids of a Human Glioma Cell Line Bearing Receptors for Platelet-Derived Growth Factor (PDGF)

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Glycolipids of U-1242 MG were characterized because results of previous studies showed that exogenous gangliosides, especially G_{M3}, inhibit PDGF-stimulated growth of this human glioma cell line. G_{M3} and G_{M2} are the major gangliosides; both separate as doublets with thin-layer chromatography. The major neutral glycolipid is glucocerebroside with nonhydroxy fatty acids, but paragloboside, ceramide dihexoside, globoside, and asialoG_{M2} (G_{A2}) are also present. The coexistence in U-1242 MG of these gangliosides and the PDGF receptor, whose mitogenic signal is modulated by G_{M3} in these cells, suggests a possible functional relationship among them with respect to growth regulation.
Lipids 27, 308-310 (1992).

Growth inhibition was one of the first described biological responses of cultured cells to exogenously added gangliosides, and it has been suggested that this may be mediated through the ability of gangliosides and their metabolites to affect protein phosphorylation by regulating several different protein kinases and phosphatases (1-3). Bremer *et al.* (4,5) have shown that gangliosides can inhibit i) growth of 3T3 fibroblasts stimulated by platelet-derived growth factor (PDGF), and ii) autophosphorylation of the receptors for PDGF and epidermal growth factor (EGF). The potency to cause these effects varied among different gangliosides, but G_{M3}, a major ganglioside in fibroblasts, was one of the most effective. U-1242 MG is a cell line derived from a human malignant astrocytoma. Its major current point of interest is that it bears receptors for PDGF (6,7) and responds mitogenically to PDGF, suggesting that PDGF may be a biological mitogen for this glioma (8). If gangliosides are biological regulators of the PDGF receptor, then it would be expected that gangliosides which have been found to be more effective in inhibiting PDGF-stimulated growth and autophosphorylation of the PDGF receptor, such as G_{M3}, might be prevalent in U-1242 MG cells. In this paper we describe the results of glycosphingolipid analyses of this cell line which show that this is the case.

MATERIALS AND METHODS

For biochemical analyses, U-1242 MG cells were seeded into 150-cm^2 plastic cell culture flasks (Corning, Corning, NY) in Eagle's minimal essential medium containing 10% calf serum at 10,000 cells/cm^2. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C; culture media were changed every third day. When cultures were subconfluent they were harvested using 0.01% trypsin in 0.01% EDTA, and before the cells were pelleted, aliquots of cell suspension were removed for total protein determinations [using the method of Bradford (9)] and for quantitation of cell numbers. Total lipids were extracted from cell pellets by homogenizing them in chloroform/methanol/water (5:5:1, by vol) and by separating the non-lipid residue by centrifugation. Ganglioside and neutral glycolipid fractions were purified using methods previously described in detail (10,11). The procedure involves an initial Folch partition, followed by three sequential washes of the lower phase with theoretical upper phase containing water but no KCl. Triplicate aliquots of washed lower phase were weighed using a Cahn microbalance. Neutral glycolipids were purified from the lower phase by silicic acid column chromatography and alkaline methanalysis. Individual neutral glycolipids were separated and quantitated using high-performance liquid chromatography (HPLC) (10). Cerebrosides were qualitatively identified on the basis of their hexose content using thin-layer chromatography (TLC) on borate impregnated silica gel plates (12,13), and detected by spraying with diphenylamine followed by heating (14). Immunostaining for galactocerebroside was performed after separating the neutral glycolipids on aluminum backed silica gel 60 high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) in the solvent system chloroform/methanol/water (65:25:4, v/v/v). This was done using the monoclonal antibody O1, provided by Dr. S.E. Pfeiffer (15), and the Avidin-Biotin alkaline phosphatase kit (Vector Laboratories, Burlingame, CA) (16). Paragloboside was detected in a similar way using the antibody F1H11 from Dainabot (Chiba, Japan) (17). The pooled upper phases were desalted using a C_{18} reversed phase column, and total gangliosides were quantitated using the resorcinol reaction (18). Gangliosides were separated using HPTLC and quantitated using scanning densitometry. The usual solvent system was chloroform/methanol/0.2% $CaCl_2$ (55:45:10, v/v/v), but to separate *N*-acetylneuraminic acid (NeuAc) from *N*-glycolyl-neuraminic acid (NeuGc) containing gangliosides, the solvent system used was chloroform/methanol/5M ammonium hydroxide/0.4% $CaCl_2.2H_2O$(50:50:4:5, by vol). Immunostaining for NeuGc-containing G_{M3} was performed as described above for galactocerebroside, but using the monoclonal antibody 2-39M (19). Antibody 2-39M, directed against NeuGc containing G_{M3}, was provided by Dr. Kenneth Lloyd. The NeuGc-containing G_{M3} standard used as a positive control was from Matreya, Inc. (Pleasant Gap, PA).

RESULTS AND DISCUSSION

The amounts of total gangliosides and neutral glycolipids in U-1242 MG cells are shown in Table 1. The values for

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Abbreviations: CDH, ceramide dihexoside; EGF, epidermal growth factor; Glob, globoside; HFA, cerebroside containing hydroxy fatty acid; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; NFA, cerebroside containing nonhydroxy fatty acid; PDGF, platelet-derived growth factor; SPG, sialosylparagloboside.

TABLE 1

Amounts of Total Gangliosides and Neutral Glycolipids in U-1242 MG Cells[a]

Gangliosides			Neutral glycolipids		
Per mg protein	Per mg total lipids	Per 10^7 cells	Per mg protein	Per mg total lipid	Per 10^7 cells
1.03 ± 0.13	8.8 ± 3.4	5.2 ± 1.1	1.1 ± 0.37	8.7 ± 1.02	6.7 ± 1.27

[a]Results for gangliosides are expressed as nmoles sialic acid, for neutral glycolipids as nmoles glycolipid. Values represent the means ± SEM for three separate experiments.

both lipid classes expressed on the basis of total lipid and total protein are within the same range previously reported for the few other human glioma cell lines studied (10,11,20-22). G_{M3} and G_{M2} were the major gangliosides making up 57% and 43%, respectively, of the total gangliosides, with only trace amounts (not quantitated) of one which migrated just behind G_{D1a} and another behind G_{D1b}. G_{M1} was not detected. Both of the major gangliosides in U-1242 MG migrated as doublets (Fig. 1). In the basic solvent system, the faster migrating of the G_{M3} doublet comigrated with G_{M3} containing *N*-acetylneuraminic acid, while the other comigrated with *N*-glycolylneuraminic acid-containing G_{M3}. However, on immuno-TLC, neither ganglioside reacted with monoclonal antibody 2-39M, which is specific for NeuGc-containing G_{M3}. Therefore, it seems most likely that these G_{M3} gangliosides separate on the basis of either their sphingosine or fatty acid composition. The G_{M2} doublet migrated as a single band in the basic system, indicating that both of the G_{M2} bands in the neutral system contain only one type of sialic acid, probably NeuAc. The proportions of these gangliosides are shown in Table 2.

This relatively simple ganglioside pattern is remarkable, because other human glioma cell lines previously reported have had significant amounts of disialogangliosides (22), and some even had trisialogangliosides (23,24). The presence of G_{M1} has been variable in previously reported human glioma cell lines, but gangliotetraose oligosaccharide cores have been present in all others studied (11,20,22-24).

The major neutral glycolipid in U-1242 MG is cerebroside which eluted on HPLC with a retention time equal to that of cerebroside containing nonhydroxy fatty acids (Table 3). It is approximately five times more prevalent than the cerebroside which coeluted with cerebroside containing hydroxy fatty acid (HFA). On immuno-TLC none of the cerebroside reacted with the O1 antibody (which reacted strongly with the galactocerebroside control). On borate impregnated TLC plates all of the cerebroside from U-1242 MG migrated with glucocerebroside. Therefore, the major neutral glycolipid in these cells is glucocerebroside, the majority of which contains nonhydroxy fatty acids. Glucocerebroside is both an anabolic precursor and a catabolic degradation product of all of the gangliosides and neutral glycolipids found in this cell line. Whether it has a biological role other than an intermediate metabolite of other glycolipids is presently unknown.

U-1242 MG cells also contain lesser amounts of ceramide dihexoside (CDH), asialo-G_{M2} (G_{A2}), and globoside (glob) (Table 3). CDH, G_{A2}, G_{M3} and G_{M2} are all closely related metabolically. Therefore it is conceivable that changes in the activities of sialytransferases and sialidases

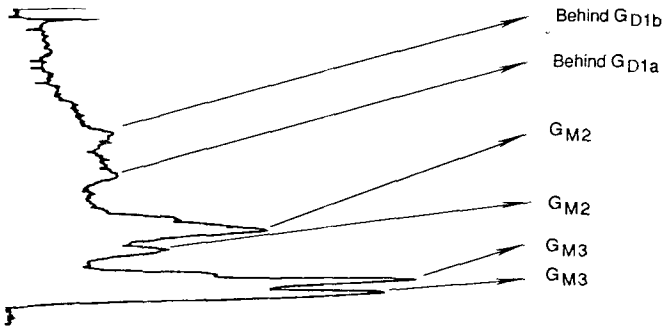

FIG. 1. Densitometric scan of gangliosides from U-1242 MG. A mixture of purified gangliosides (1 μg sialic acid) isolated from U-1242 MG was spotted on an HPTLC plate and developed in chloroform/methanol/0.2% $CaCl_2$ (55:45:10, v/v/v). The plate was air-dried, sprayed with the resorcinol spray, and fractions were made visible by heating on an aluminum plate at 95°C. The HPTLC plate was scanned using a Shimadzu CS-910 scanning densitometer (Shimadzu, Tokyo, Japan).

TABLE 2

Percentage Distribution of Gangliosides in U-1242 MG Cells[a]

G_{M3}		G_{M2}	
Upper	Lower	Upper	Lower
25.0 ± 5.0	32.3 ± 4.6	11.7 ± 1.7	30.9 ± 2.9

[a]Values are based on the integration units of the areas under the peaks for at least five scans for each of three samples. Upper and lower refer to the relative migrations of the components of the doublets for each ganglioside separated by high-performance thin-layer chromatography.

involved in their interconversions could modulate the functions of the PDGF receptor by altering the concentrations of these glycolipids in the plasmalemma. Paragloboside migrated as a triplet on both HPLC and HPTLC with approximately equimolar amounts in each band. Its identity was proven further by visualizing all three bands on immuno-TLC with the antibody F1H11. One major difference between the neutral glycolipids of this human glioma cell line and the other four described in the literature (10) is the presence of modest amounts of paragloboside in U-1242 MG which was not detected in any of the others. However, immunodetection was not used previously. The relationship of the neutral glycolipid composition to the function of the PDGF receptor, if any,

TABLE 3

Neutral Glycolipid Composition of U-1242 MG[a]

Glycolipid	Per mg protein	Per mg total lipid	Per 10^7 cells	Molar %
NHF cerebroside	0.45 ± 0.16	3.6 ± 0.51	2.7 ± 0.71	40
HFA cerebroside	0.01 ± 0.032	0.80 ± 0.09	0.60 ± 0.14	8.9
CDH	0.17 ± 0.06	1.35 ± 0.24	1.0 ± 0.29	15.0
G_{A2}	0.037 ± 0.015	0.27 ± 0.081	0.23 ± 0.088	3.4
Globoside	0.11 ± 0.034	0.84 ± 0.09	0.66 ± 0.18	10.0
Paragloboside	0.247 ± 0.0081	1.91 ± 0.25	1.50 ± 0.44	23.0

[a]Values are means ± SEM for three separate experiments and represent nmoles of each glycolipid. At least three chromatograms were obtained for each specimen, and values were averaged.

is unknown. Although sphingosine is an inhibitor of protein kinase C (reviewed in ref. 25), there is little evidence that any neutral glycolipid has an effect on the activity of any protein kinase.

G_{M3}, which comprises 57% of the total ganglioside in U-1242 MG cells, is a relatively potent inhibitor of both PDGF-stimulated growth of U-1242 MG (8) and Swiss 3T3 fibroblasts (4). G_{M3} also inhibits autophosphorylation of the PDGF receptor (4). Of relevance to the present study is the finding that sialosylparagloboside (SPG) was inactive in both respects (4). Although paragloboside is present in U-1242 MG, no SPG was found. The ganglioside composition reported here is compatible with the possibility that G_{M3} could play a role in regulating the degree of autophosphorylation of the PDGF receptor, thus modulating signal transduction of the PDGF signal for mitogenesis in U-1242 MG cells.

The pattern of gangliosides reported here for U-1242 MG cells has not been seen in all human glioma lines studied so far, nor have PDGF receptors been found in all glioma cell lines examined (6,7). Thus, it is possible that glioma cells whose growth is influenced by PDGF may have ganglioside and neutral glycolipid contents similar to those described here for U-1242 MG. If this proves to be the case, then the glycolipid composition of glioma cells could play a role in their growth by modulating the function of the PDGF receptor and, thus, might be a marker for cells which interact with PDGF. This would have implications of both biological and clinical importance, and the U-1242MG cell line could be used as a model system to investigate the molecular mechanisms involved in the interactions of ganglioside with the PDGF receptor in human glioma cells.

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Liver Lipid Profiles of Adults Taking Therapeutic Doses of Aspirin

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The distributions of lipids of hepatic specimens obtained at autopsy from 7 adult patients who had been taking large amounts of aspirin for arthritis were compared to 7 control samples obtained from livers of autopsied adults without prior liver disease. The total neutral lipid levels of control livers were approximately one-third lower than those observed for livers of patients on aspirin. In addition, the phospholipid content of control specimens was significantly greater than that of livers from adult patients that had been on a high dose of aspirin for a long time. Examination of individual lipid classes showed that the concentrations of free fatty acids, triacylglycerols, and mono- and diacylglycerols were highest in livers of patients with aspirin exposure, and that all phospholipids were diminished. Phosphatidylcholines and phosphatidylethanolamines showed the greatest decrease. These results suggest that the livers of patients taking large amounts of aspirin may accumulate fatty acids and neutral lipids due to an impairment in the oxidation of fatty acids by hepatocytes. The data obtained also suggest that needle biopsy of the liver with measurement of distribution of hepatic lipids, perhaps together with histopathologic examination, may provide useful diagnostic information.
Lipids 27, 311-314 (1992).

Acetylsalicylic acid (aspirin) has been shown to affect the liver adversely under a number of clinical conditions. High-dose aspirin can elevate liver transaminases in patients with rheumatoid arthritis and systemic lupus erythematosis (1). Aspirin inhibits not only cyclooxygenase but also several other enzymes involved in respiratory burst and in lipid mobilization (2,3). Biopsies show mononuclear cell infiltration of the portal triads with occasional focal necrosis, but the lipid compositions of such hepatic tissues have not been analyzed previously.

In addition to aspirin toxicity in rheumatoid arthritis, aspirin has been implicated in the development of Reye's syndrome. Although this condition has been extensively studied since it was first described, the etiology and pathogenesis of the syndrome remain unclear. Mitochondrial changes in various organ systems, including the liver, brain, kidney, skeletal muscle, pancreas, and myocardium have been reported (4).

Other syndromes that have been ascribed to high aspirin intake (5) are characterized by lowered activities of mitochondrial enzymes involved in ureagenesis (orithine transcarbamylase and carbamylphosphate synthetase) and gluconeogenesis (pyruvate carboxylase), and of enzymes associated with the tricarboxylic acid cycle (citrate synthetase, glutamate dehydrogenase, succinic dehydrogenase, malic dehydrogenase, and isocitrate dehydrogenase). Further evidence of mitochondrial injury derives from the occurrence of dicarboxylic acids in both serum and urine in several disorders including dicarboxylic aciduria, isovaleric acedemia, and others (6-9).

Biochemical abnormalities in lipid metabolism may account for some of the pathogenic features of this condition. Stimulated lipolysis from adipose tissues with impaired hepatic oxidation of mobilized fatty acids and panlobular microvesicular fatty changes have been found in various stages of Reye's syndrome and in alcoholics (10-13). Also, some patients taking therapeutic doses of aspirin tend to be hypoketotic, and dicarboxylic acids can be detected in serum and urine specimens. These metabolic derangements may suggest compromised hepatic mitochondrial β-oxidation of fatty acids.

This study was undertaken to characterize and quantify the major lipid classes found in the liver of patients taking high-dose aspirin and to compare with those found in adult control specimens obtained at autopsy. The study indicates that the pattern of distribution of hepatic lipids found in adult patients on high-dose aspirin differs greatly from that observed in liver specimens obtained from adult control subjects which points toward a metabolic impairment in hepatic mitochondrial fatty acid oxidation. The results obtained also show a striking similarity between the lipid distribution in livers of adults on high-dose aspirin and reported hepatic lipid distributions in children with Reye's syndrome.

MATERIALS AND METHODS

Patients. Liver biopsies were obtained from seven patients with severe rheumatoid arthritis who had been taking between 3.25 and 5.85 g of aspirin daily for many years. All patients died from myocardial infarctions, had no history of ethanol intake, and had no known liver function abnormalities at the time of death. Patients had no other known medical problems and none were obese. Controls biopsy specimens were obtained from age-matched patients who also died of myocardial infarction but had not been on aspirin and likewise had no known liver disease or ethanol abuse history. T.G.V. and A.P.A. selected the patients and obtained 1-2 g biopsies from the center of the liver 1-2 days after the demise of the patient.

Lipid assays. Lipid standards were purchased from Applied Sciences (State College, PA) and were tested for purity by thin-layer chromatography (TLC) and gas-liquid chromatography. The liver specimens were extracted twice with 100 mL of chloroform/methanol (2:1, v/v); additional extractions did not yield additional lipid material. The extracts were washed with 5 mL of 0.1 M KCl solution (14).

One aliquot of the extract was subjected to silicic acid (BioSil A, 100-200 mesh) column chromatography to separate the neutral lipids (by chloroform elution) from the phospholipids (by methanol elution). Individual lipid classes were obtained from the neutral lipids and from the phospholipids by TLC. Silica Gel G plates were developed with petroleum hydrocarbon/diethyl ether/glacial acetic

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Abbreviations: MCAD, medium chain acylcoenzyme A dehydrogenase; SEM, standard error of the mean; TLC, thin-layer chromatography.

acid (90:10:1, v/v/v) for the separation of neutral lipids. Phospholipids were separated by TLC on Silica Gel H plates using chloroform/methanol/acetic acid/water (200:120:25:15, by vol) as developed solvent. Known amounts of authentic standards were applied and developed alongside each experimental sample. Iodine vapor was used to localize lipid spots. The stained areas were then scraped from the plates and transferred to individual test tubes containing 2 mL of concentrated sulfuric acid. The tubes were heated at 200°C for 15 min, cooled by immersion into ice, and 3 mL of water was added. Following centrifugation for 5 min at 3000 rpm to remove the silica gel, each solution was decanted into a quartz cuvette. The charred lipids were quantitatively assayed by use of a Beckman DB Spectrometer (Beckman, Fullerton, CA) set at 375 nm by comparison with known standards treated in the same manner. The sum of the data from all charred fractions was defined to total 100%. Other details of the methods used for lipid assay have been described previously (15).

Total lipid content of control cases was 65.0 ± 1.3 mg/g wet weight of liver. The total lipid content of the specimens obtained from the liver of patients that had been taking a high dose of aspirin was 80.1 ± 3.1 mg/g wet weight.

RESULTS AND DISCUSSION

The lipid distribution of liver specimens obtained at autopsy from seven rheumatoid patients that had been taking a high dose of aspirin are shown in Tables 1–4. Specimens were also obtained from livers of seven control subjects who showed no evidence of any disease affecting the liver.

Examination of the total lipids extracted from livers of control subjects compared to those obtained from specimens of rheumatoid patients on high dose aspirin revealed that about half the total lipids from controls comprised neutral lipids and half comprised phospholipids (Tables 1 and 3). By contrast, approximately two-thirds of the lipids found in high aspirin specimens were neutral lipids whereas only about one-third were phospholipids (Tables 2 and 4).

Table 1 shows the distribution of neutral lipids extracted from liver tissue specimens obtained from control patients. The mean of total neutral lipids was 49.5 ± 1.0% which differs markedly from the mean of 65.6 ± 0.7% for rheumatoid patients on high-dose aspirin (Table 2). A most striking difference was observed in the hepatic content of free fatty acids from the controls (12.6 ± 1.5%) which was less than half that (27.4 ± 2.4%) for rheumatoid patients on high-dose aspirin. The data in Table 2 also describe the neutral lipids for each specimen from patients on high-dose aspirin. The data illustrate the greater accumulation of hepatic neutral lipids in these patients compared to control subjects.

In Tables 3 and 4 we present the phospholipid compositions of specimens of liver tissue obtained from control subjects and from rheumatoid patients on high-dose

TABLE 1

Neutral Lipid Composition of Liver Tissue of Control Subjects[a]

Neutral lipids	Case 1	2	3	4	5	6	7	Mean ± SEM[b]
Free fatty acids	11.3	12.2	9.2	9.5	14.5	16.3	10.4	12.6 ± 1.5
Mono- and diacylglycerols	2.0	2.1	2.2	2.4	2.2	2.3	2.8	2.3 ± 0.1
Triacylglycerols	13.8	12.3	11.7	12.6	11.6	8.8	12.4	11.9 ± 0.6
Fatty acid esters	3.5	3.4	2.5	2.8	3.1	4.1	3.4	3.3 ± 0.2
Cholesterol	7.7	9.2	6.5	8.8	6.9	8.6	8.6	8.0 ± 0.4
Cholesteryl esters	7.5	5.7	7.3	8.9	7.0	3.8	7.4	6.8 ± 0.6
Undetermined	4.9	5.1	4.1	5.2	5.0	6.1	7.0	5.3 ± 0.4
Total neutral lipids	50.7	50.0	43.5	50.2	50.3	50.0	52.0	49.5 ± 1.0

[a]Percent distribution of total lipids extracted. [b]Standard error of the mean.

TABLE 2

Neutral Lipid Composition of Liver Tissue Obtained from Patients on High-Dose Aspirin[a]

Neutral lipids	Case 1	2	3	4	5	6	7	Mean ± SEM[b]
Free fatty acids	15.0	35.0	32.0	27.3	26.4	30.6	25.3	27.4 ± 2.4
Mono- and diacylglycerols	7.8	3.0	5.0	3.0	8.0	8.6	9.2	6.4 ± 1.0
Triacylglycerols	30.1	10.5	12.0	9.0	7.4	4.1	11.2	12.0 ± 3.2
Fatty acid esters	3.0	4.0	5.0	5.0	5.1	6.4	5.2	4.8 ± 0.4
Cholesterol	3.0	5.0	5.0	8.0	6.5	8.1	5.7	5.9 ± 0.7
Cholesteryl esters	3.0	5.0	6.0	7.0	6.0	6.9	7.2	5.9 ± 0.6
Undetermined	2.0	3.5	3.0	4.7	4.0	2.5	2.9	3.2 ± 0.4
Total neutral lipids	63.9	66.0	68.0	64.0	63.4	67.2	66.7	65.6 ± 0.7

[a]Percent distribution of total lipids extracted. [b]Standard error of the mean.

TABLE 3

Composition of Liver Tissue of Control Subjects[a]

Phospholipids	Case 1	2	3	4	5	6	7	Mean ± SEM[b]
Phosphatidylinositols	3.1	3.6	3.0	3.0	3.3	2.4	2.9	3.0 ± 0.1
Phosphatidylethanolamines	13.1	12.8	15.8	12.9	12.5	14.2	13.1	13.5 ± 0.4
Phosphatidylserines	7.0	4.9	4.0	3.7	4.1	4.8	3.7	4.6 ± 0.4
Phosphatidylcholines	15.1	12.3	20.0	13.1	14.1	11.2	15.1	14.4 ± 1.1
Lysophosphatidylcholines	1.0	1.2	0.7	1.0	1.4	1.0	1.0	1.0 ± 0.1
Cardiolipins	0.5	0.6	0.5	0.4	0.2	0.7	1.0	0.6 ± 0.1
Phosphatidic acids	5.1	9.7	9.0	12.0	9.9	10.2	8.3	9.2 ± 0.8
Sphingomyelins	3.2	4.5	2.9	2.2	2.8	2.1	2.0	2.8 ± 0.3
Undetermined	1.2	0.4	0.6	1.5	1.4	3.4	0.9	1.3 ± 0.4
Total phospholipids	49.3	50.0	56.5	49.8	49.7	50.0	48.0	50.5 ± 1.0

[a]Percent distribution of total phospholipids extracted. [b]Standard error of the mean.

TABLE 4

Phospholipid Composition of Liver Tissue Obtained from Patients on High-Dose Aspirin[a]

	Case 1	2	3	4	5	6	7	Mean ± SEM[b]
Phosphatidylinositols	2.4	1.7	2.5	3.1	2.0	1.6	2.5	2.3 ± 0.2
Phosphatidylethanolamines	13.6	7.4	6.0	7.1	3.0	2.4	2.7	6.0 ± 1.5
Phosphatidylserines	2.0	3.2	3.0	4.0	4.0	3.1	3.5	3.3 ± 0.3
Phosphatidylcholines	6.0	5.5	5.0	4.3	7.0	6.8	7.3	6.0 ± 0.4
Lysophosphatidylcholines	1.0	0.7	1.0	1.0	1.5	1.0	1.4	1.1 ± 0.1
Cardiolipins	0.3	0.4	0.2	0.3	0.1	0.2	0.3	0.3 ± 0.0
Phosphatidic acids	7.0	8.3	13.1	13.0	16.0	13.0	12.5	11.8 ± 1.2
Sphingomyelins	2.0	6.3	1.0	.8	2.0	2.7	1.1	2.3 ± 0.7
Undetermined	1.8	0.5	0.2	0.4	1.0	2.0	2.0	1.1 ± 0.3
Total phospholipids	36.1	34.0	32.0	34.0	36.6	32.8	33.3	34.1 ± 0.6

[a]Percent distribution of total phospholipids extracted. [b]Standard error of the mean.

aspirin, respectively. The mean percentage of hepatic phospholipids for patients on high-dose aspirin was 34.1 ± 0.6% while that for controls indicated a much higher phospholipid content, namely 50.5 ± 1.0%. The largest disparity in phospholipid subclasses was observed for phosphatidylethanolamine (6.0 ± 1.5% in patients *versus* 13.5 ± 0.4% in controls) and for phosphatidylcholine (6.0 ± 0.4% in patients *versus* 14.4 ± 1.1% in controls). There also was a dramatic decline in cardiolipin levels for patient samples (0.3 ± 0%) which was only half that found in controls (0.6 ± 0.1%). The other phospholipid subclasses did not differ significantly between the two groups of specimens studied.

In the present study, lipid analyses of hepatic autopsy specimens from rheumatoid patients who had been taking high doses of aspirin have indicated significant differences from those of similar specimens from autopsied control subjects. The data suggest that a major metabolic impairment of fatty acid oxidation may exist in patients taking a high dose of aspirin. The increased neutral lipids and free fatty acids found in the liver specimens from these patients would be consistent with decreased oxidative capacity.

Mitochondrial morphologic changes, metabolic changes, and a clinical presentation similar to rheumatoid patients taking a high dose of aspirin have previously been described for patients with medium-chain acyl-coenzyme A dehydrogenase (MCAD) deficiency, an inherited defect in mitochondrial β-oxidation of fatty acids (16). This supports the notion that a relationship may exist between abnormalities in fatty acid oxidation and the observed clinical signs and symptoms.

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ARTICLE

The Effect of Borage Oil Consumption on the Composition of Individual Phospholipids in Human Platelets

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The effect of supplementation with borage oil containing γ-linolenic acid (GLA, 18:3n-6) on the levels and fatty acid compositions of individual human platelet phospholipids was evaluated. For this purpose, male volunteers were given an average daily intake of 5.23 g of GLA (as borage oil) for 42 days, after which the supplement was withdrawn for an additional 42-day period. No significant differences were found in the relative amounts of the choline phospholipids (PC), ethanolamine phospholipids (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SPH) at days 0, 22, 43, 64, and 85. However, marked differences were observed in the fatty acid compositions of all the phospholipids including a marked, and reversible, rise in the level of dihomo-γ-linolenic acid (DGLA, 20:3n-6), without a significant elevation in arachidonic acid (AA, 20:4n-6) and decreases in n-3 polyunsaturated fatty acids. In the case of PC, a net rise in DGLA of 1.8 mol% was observed by day 22 (from 2.1 to 3.9 mol%). The DGLA/AA ratios at day 43 exhibited considerable variability across phospholipids with PC > PS > PE = PI; the PC, PE, PS, and PI accounted for 67.6, 16.7, 12.9, and 2.6%, respectively, of the total DGLA in platelet phospholipids. Interestingly, despite the lack of DGLA in SPH, this phospholipid exhibited a marked enrichment in nervonic acid (NA, 24:1n-9) from 16.2 to 24.7 mol% upon borage oil consumption. The observed alterations may represent biochemical strategies for adaptation to dietary fatty acid modifications and the regulation of platelet membrane functioning.
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The activation of human platelets plays an intimate role in the sequence of events leading to arterial thrombosis and atherosclerotic cardiovascular disease (for reviews, see refs. 1–4). In this regard, the fatty acid compositions of platelet phospholipids are of particular interest since arachidonic acid (AA, 20:4n-6), upon release *via* phospholipase activities in stimulated platelets, is converted into thromboxane A_2 (TxA_2), a potentiator of platelet aggregation and a vasoconstrictor compound (5,6). With respect to nutritional modification of platelet fatty acid composition and prevention of arterial thrombosis, considerable interest in fish/fish oils containing n-3 polyunsaturated fatty acids has arisen (7,8). Both dietary eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (9) have been shown to have the ability to partially replace AA and other unsaturated fatty acids in various human platelet membrane phospholipids. Separation of the individual phospholipids from the platelets of humans consuming n-3 polyunsaturated fatty acids has revealed considerable individual variability in the degree of EPA and DHA enrichment, including AA replacement (10–13). For example, phosphatidylinositol (PI) is greatly enriched in AA and resilient to replacement of AA by EPA in contrast to choline (PC) and ethanolamine phospholipids (PE).

Recently, there has been considerable interest in the potential health benefits of dietary supplementation with γ-linolenic acid (GLA, 18:3n-6) found in evening primrose oil (14), black currant seed oil (15), and borage oil (16). In the latter oil, the level of GLA is approximately 22% which greatly surpasses the levels in evening primrose and black currant seed oil. The ratio of GLA/linoleic acid is approximately 7-fold that present in primrose oil.

The consumption of GLA can provide for the enrichment of cells and tissues including platelets in dihomo-γ-linolenic acid (DGLA, 20:3n-6) (14–19) derived *via* fatty acid elongation. The suggested health benefits of consuming dietary GLA are based on a potentially increased availability of DGLA, without an accompanying rise in the Δ5 desaturase product, AA (20). Interest in increasing the DGLA/AA ratio in mammalian cells is based on the expected increase in the biosynthesis of PGE_1 relative to the AA derived eicosanoids, including TxA_2, in terms of platelet reactivity (20, 21). *In vitro* studies have indicated that PGE_1 has a significant anti-aggregatory effect (20,21). Whereas some information is available on the effect of dietary supplementation with DGLA or evening primrose oil containing GLA on the fatty acid composition of total or selected platelet phospholipids found in animal models (22,23) and in isolated human trials (17,24,25), no data are available in the literature on the effect of dietary GLA on the fatty acid composition of the various phospholipids including sphingomyelin (SPH) found in the platelets of human volunteers. SPH was of particular interest since the EPA/DHA enrichment of platelet phospholipids observed in human subjects ingesting fish oil was accompanied by a significant rise in nervonic acid (NA, 24:1n-9) in SPH (10).

MATERIALS AND METHODS

Subjects and experimental design. The subjects were 6 healthy male volunteers with a mean age of 33 years and a body weight of 73.1 kg. They claimed to have abstained from medication, alcohol, tobacco, and fish, and to have otherwise maintained their normal dietary habits from 2 wk prior to day 0 up to day 85. The Human Ethics Committee of the University of Guelph gave certification for the study and the subjects signed an informed consent.

The borage oil capsules (PGE Canada, Saskatoon, Canada) were consumed on a per kg body weight basis (71.6 mg of GLA/kg/day) for 42 consecutive days starting on day 1, after which the supplement was removed for a subsequent 42-day period. The borage oil capsules each contained 1 g of oil (21.8 wt% GLA and 10 IU of α-tocopherol) and were consumed on average in the amount of

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NA, nervonic acid; PC, choline phospholipids; PE, ethanolamine phospholipids; PI, phosphatidylinositol; PRP, platelet-rich plasma; PS, phosphatidylserine, SPH, sphingomyelin; Tx, thromboxane.

24 capsules/day which provided the equivalency of 5.23 g (mean intake) of GLA daily. No significant change was found in the percent GLA in the encapsulated borage oil during storage based on routine analysis. The subjects gave blood for platelet isolation on consecutive days 0, 22, 43, 64, and 85.

Platelet isolation. Approximately 70 mL of blood were drawn from the antecubital veins into siliconized vacutainer tubes containing 1/6 volume of acid-citrate-dextrose (ACD) as an anticoagulant (26). Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 111 × *g* for 15 min at 30°C. PRP was then centrifuged at 2000 × *g* for 15 min at 37°C. The derived platelet pellet was suspended in platelet suspension I (PS-I) consisting of Tyrodes buffer (NaCl, 137 mM; KCl, 2.7 mM; $NaHCO_3$, 12 mM; NaH_2PO_4, 0.4 mM; MgCl, 1 mM; $CaCl_2$, 2mM; glucose, 5.5 mM; pH 7.35; osmolarity, 290–300 mOsm; 0.35% albumin; HEPES, 2.5 mM), heparin (50 units/mL) and apyrase (30 μg/mL). PS-I was incubated for 10 min at 37°C and then centrifuged at 1200 × *g* for 10 min (27). The latter platelet pellet was suspended in Tyrodes buffer, and washed platelet suspensions were prepared according to Skeaff and Holub (28) based on the method of Mustard *et al.* (27). Final platelet concentrations were adjusted to 2×10^9/mL with Tyrodes buffer (see above) using a Coulter counter (Model ZM, Coulter Electronics of Canada, Ltd., Burlington, Ontario, Canada).

Platelet phospholipid analyses. Platelets were extracted according to Bligh and Dyer (29) using 3.75 mL of chloroform/methanol (1:2, v/v), 1.25 mL of chloroform, and 1.25 mL of water for 1 mL of platelet suspension. The methods for the thin-layer chromatographic separation of the individual phospholipids and determination of their fatty acid compositions by gas-liquid chromatography were as described (30). Conversions of the determined fatty acid amounts (mols) into phospholipid employed factors of 0.5, 0.75, 0.5, 0.5, and 1.0 in the case of PC, PE, phosphatidylserine (PS), PI, and SPH, respectively. There are approximately equal amounts of diacyl and alkenylacyl PE; as well up to 94% of the PC has been reported to be diacyl (31). This gives a rounded conversion factor of 0.5 for PC.

Statistical analyses. The data were assessed with a randomized complete block design using Tukey's Honestly Significant Difference test (32) to determine individual differences across various sampling times. The level of significance was selected at $P < 0.05$.

RESULTS

The fatty acid composition of the borage oil is shown in Table 1. The major fatty acids were oleic, linoleic (LA, 18:2n-6), and GLA. The wt percentage of GLA was 21.8% which agreed very well with the manufacturer's data claiming 22.0% (PGE Canada).

Borage oil consumption for 42 days did not change the relative contributions of the individual phospholipids to the total over the periods of consideration (see Fig. 1).

The fatty acid compositions of all the individual phospholipid classes were changed significantly by borage oil supplementation (Tables 2–6). In the case of PC (Table 2), there was a notable rise ($p < 0.05$) in DGLA which reached 3.9 mol% (from 2.1 mol%) by day 22. A rise in GLA (from 0.07 to 0.3 mol%) was also noted. Interestingly,

TABLE 1

The Fatty Acid Composition of Encapsulated Borage Oil[a]

Fatty acids	Wt% of total
16:0	9.7 ± 0.1
18:0	4.2 ± 0.1
18:1	16.3 ± 0.1
18:2n-6	35.5 ± 0.2
18:3n-6	21.8 ± 0.1
18:3n-3	0.2 ± 0.0
18:4n-3	0.1 ± 0.02
20:0	0.3 ± 0.0
20:1	4.8 ± 0.2
20:2n-6	0.5 ± 0.1
22:0	0.2 ± 0.0
22:1	2.9 ± 0.1
24:0	< 0.1
24:1n-9	1.8 ± 0.04

[a]The values represent the means ± SEM (n = 5) of randomly selected capsules.

there was no change in AA while a moderate reduction in LA was observed. Conversely, there was a statistically significant decrease in the level of the n-3 polyunsaturated fatty acids including α-linolenic acid (ALA, 18:3n-3), and EPA with no significant decrease in DHA. This was manifested in an increased ratio of DGLA/AA. The aforementioned alterations attained by day 22 showed no further change at day 43; these fully reverted to entry values by day 85.

In the case of PE (Table 3), PS (Table 4), and PI (Table 5), similar trends in DGLA were seen as for PC (Table 2) but to varying degrees. A decrease in DHA was observed in PE and PS following borage oil consumption for 42 days (Tables 3 and 4). In terms of ratios, the DGLA/AA ratio rose from 0.02 to 0.05 in the case of PE (Table 3), 0.1 to 0.2 (PS) (Table 4), and 0.01 to 0.05 (PI) (Table 5).

Interestingly, SPH (Table 6) contained no detectable AA at day 0 and, upon supplementation with borage oil containing GLA, no enrichment of this phospholipid in DGLA nor AA accumulation was found. However, there was a substantial enrichment of this phospholipid in NA without an accompanying rise in other monoenes (18:1 and 22:1) at day 22. Cessation of borage oil supplementation returned the NA levels to entry values.

It was determined (Fig. 2) that 67.6% of the DGLA in phospholipid resided within PC with PE, PS, and PI accounting for 16.7, 12.9, and 2.6%, respectively.

DISCUSSION

The consumption of borage oil containing GLA significantly affected the fatty acid compositions of the n-6 plus n-3 series and DGLA/AA ratios of the individual phospholipids of human platelets. However, it did not alter the relative levels of the individual phospholipid classes as a percentage of the total phospholipid. The DGLA/AA ratios varied across the phospholipids (Tables 2–5) with PC > PS > PE = PI. The level of GLA consumption averaged approximately 1.8% of daily calories. Normal human intakes are approximately 38% of calories as fat in North America currently. In corresponding trials with fish oils containing n-3 polyunsaturated fatty acids (EPA and DHA), no corresponding alterations in the dis-

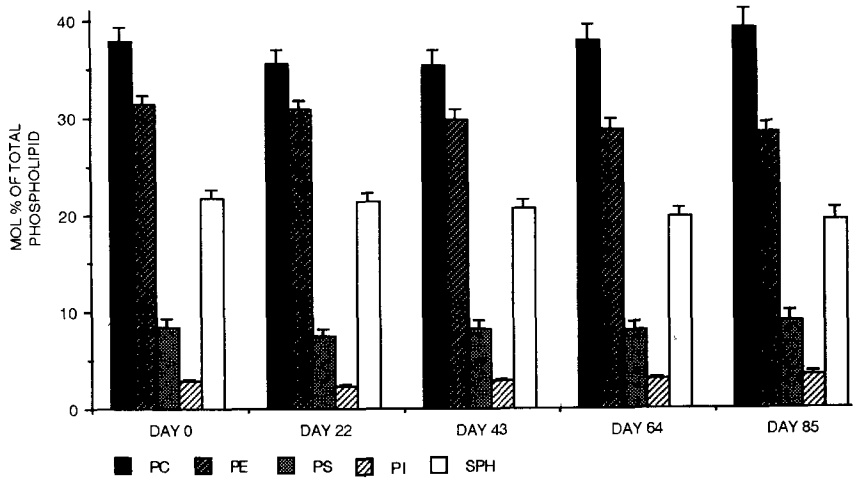

FIG. 1. Percentage mass contributions of individual platelet phospholipids before, during, and after borage oil consumption. Values are means ± SEM n = 6). No significant differences were found for a given phospholipid at all time points ($p > 0.05$).

TABLE 2

The Fatty Acid Composition of PC in the Platelets of Subjects Before, During and After Consuming Borage Oil[a]

Fatty acids	Day 0	Day 22	Day 43	Day 64	Day 85
			(mol% of total)		
16:0	27.9 ± 0.6^{b}	29.1 ± 0.8^{b}	27.6 ± 0.9^{b}	31.2 ± 0.8^{c}	30.5 ± 0.8^{b}
18:0	14.8 ± 0.5^{b}	14.2 ± 0.5^{b}	14.9 ± 0.6^{b}	13.9 ± 0.4^{c}	13.9 ± 0.4^{c}
18:1	22.7 ± 0.4^{b}	20.1 ± 0.5^{c}	18.7 ± 0.4^{c}	20.7 ± 0.2^{b,d}	22.4 ± 0.5^{b}
18:2n-6	7.9 ± 0.4^{b}	6.5 ± 0.3^{c,d}	5.9 ± 0.3^{c}	7.4 ± 0.8^{b,d}	7.7 ± 0.6^{b}
18:3n-6	0.07 ± 0.00^{b}	0.3 ± 0.10^{c}	0.2 ± 0.00^{c}	0.01 ± 0.00^{b}	0.08 ± 0.00^{b}
18:3n-3	0.10 ± 0.02^{b}	0.05 ± 0.00^{c}	0.03 ± 0.00^{c}	0.05 ± 0.00^{c}	0.1 ± 0.00^{b}
20:0	1.3 ± 0.2^{b}	1.3 ± 0.1^{b}	1.4 ± 0.2^{b}	1.2 ± 0.2^{b}	1.0 ± 0.1^{c}
20:1	1.6 ± 0.2^{b}	1.7 ± 0.1^{b}	1.6 ± 0.1^{b}	1.1 ± 0.3^{c}	1.3 ± 0.1^{b}
20:2n-6	0.6 ± 0.1^{b}	0.5 ± 0.1^{b}	0.5 ± 0.0^{b}	0.5 ± 0.1^{b}	0.6 ± 0.0^{b}
20:3n-6	2.1 ± 0.2^{b}	3.9 ± 0.6^{c}	3.8 ± 0.7^{c}	2.0 ± 0.3^{b}	2.0 ± 0.2^{b}
20:4n-6	13.5 ± 0.4^{b}	13.1 ± 0.5^{b}	12.9 ± 0.7^{b}	13.3 ± 0.3^{b}	13.5 ± 0.5^{b}
20:5n-3	0.2 ± 0.0^{b}	0.1 ± 0.0^{c}	0.08 ± 0.0^{c}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
22:6n-3	0.6 ± 0.1^{b}	0.5 ± 0.1^{b}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.6 ± 0.0^{b}
Ratios					
20:3n-6/20:4n-6	0.2 ± 0.03^{b}	0.3 ± 0.05^{c}	0.3 ± 0.04^{c}	0.1 ± 0.02^{b}	0.2 ± 0.02^{b}

[a]Borage oil consumption occurred consecutively from days 1 to 42 inclusive. Values are means ± SEM for six subjects. Values across a given row with different superscript letters are significantly different ($P < 0.05$). Other minor fatty acids have been omitted from the table.

tributions of individual phospholipids were found in platelets (10).

The fatty acid composition of each of the individual phospholipids at day 0 (Tables 2–6) was similar to literature values for healthy subjects (10,33). The increased GLA and DGLA in PC was apparently due to the consumption of high amounts of GLA and its metabolism *via* fatty acid elongation to DGLA (16,34). The noted (see Results) diminution in n-3 polyunsaturated fatty acid levels (EPA and DHA) in platelet phospholipids may reflect a decreased conversion of α-linolenic acid (ALA) to DHA by desaturation/elongation reactions. The decreased levels of n-3 fatty acids, including ALA, and n-6 fatty acids, *e.g.* LA in PC, may also reflect their replacement by DGLA in phospholipids *via* deacylation/reacylation. Interestingly, the AA-rich phospholipid PI was more resilient to enrichment with DGLA upon borage oil consumption compared to PC. The net mol% rise in DGLA, day 43 relative to day 0, averaged 1.2 mol% in the case of PI compared to 1.7, 1.1, and 1.2% in PC, PE, and PS, respectively. Whether this discrimination reflects the availability of DGLA to phospholipid biosynthetic reactions in the megakaryocyte or selectivity in the enzymic reactions involved remains to be investigated. Previous studies with dietary fish oils containing n-3 fatty acids have shown a marked resilience of platelet PI with respect to EPA entry (10,35).

The observation that AA levels in the various individual phospholipids (PC, PE, PS, PI) were unchanged upon borage oil ingestion may reflect in part the reported low activity of the Δ5 desaturase activity in human platelets (36) and a very limited exchange of AA-containing phos-

TABLE 3

The Fatty Acid Composition of PE in the Platelets of Subjects Before, During and After Consuming Borage Oil[a]

Fatty acids	Day 0	Day 22	Day 43	Day 64	Day 85
			(mol% of total)		
16:0	5.2 ± 0.8[b]	4.6 ± 0.7[b]	5.5 ± 0.6[b]	4.9 ± 0.2[b]	5.7 ± 0.4[b]
18:0	21.3 ± 1.8[b]	20.5 ± 0.8[b]	23.6 ± 1.8[b]	21.2 ± 0.6[b]	22.5 ± 0.6[b]
18:1	8.0 ± 0.5[b]	7.8 ± 0.7[b]	7.4 ± 0.6[b]	7.9 ± 0.6[b]	8.7 ± 0.5[b]
18:2n-6	2.4 ± 0.2[b]	2.3 ± 0.1[b]	2.0 ± 0.1[b]	2.6 ± 0.2[b]	2.4 ± 0.2[b]
18:3n-3	0.07 ± 0.00[b]	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.01 ± 0.01[b]	0.04 ± 0.02[b]
20:0	1.0 ± 0.3[b]	0.7 ± 0.1[b]	1.0 ± 0.1[b]	0.8 ± 0.1[b]	0.8 ± 0.1[b]
20:1	0.6 ± 0.1[b]	0.7 ± 0.0[b]	0.6 ± 0.1[b]	0.6 ± 0.1[b]	0.6 ± 0.0[b]
20:2n-6	0.1 ± 0.1[b]	0.3 ± 0.0[b]	0.2 ± 0.0[b]	0.3 ± 0.1[b]	0.4 ± 0.1[c]
20:3n-6	0.8 ± 0.3[b]	1.8 ± 0.5[b]	1.9 ± 0.4[c]	0.8 ± 0.2[b]	0.9 ± 0.1[b]
20:4n-6	42.8 ± 3.5[b]	44.4 ± 2.6[b]	38.0 ± 3.2[b]	44.8 ± 1.5[b]	44.7 ± 1.5[b]
20:5n-3	0.2 ± 0.1[b]	0.08 ± 0.0[b]	0.00 ± 0.00[c]	0.12 ± 0.00[b]	0.2 ± 0.1[b]
22:4n-6	5.7 ± 1.8[b]	9.4 ± 1.0[c,d]	7.8 ± 1.1[b,d]	7.8 ± 1.1[b,d]	7.0 ± 0.9[b,d]
22:6n-3	2.8 ± 0.4[b]	2.6 ± 0.1[b,c]	1.9 ± 0.2[c]	2.5 ± 0.1[b,c]	2.2 ± 0.1[b,c]
Ratios					
20:3n-6/20:4n-6	0.02 ± 0.007[b]	0.04 ± 0.011[c]	0.05 ± 0.010[c]	0.02 ± 0.005[b]	0.02 ± 0.009[b]

[a]See legend for Table 2.

TABLE 4

The Fatty Acid Composition of PS in the Platelets of Subjects Before, During and After Consuming Borage Oil[a]

Fatty acids	Day 0	Day 22	Day 43	Day 64	Day 85
			(mol% of total)		
16:0	1.0 ± 0.2[b,c]	1.0 ± 0.2[b,c]	0.7 ± 0.2[b]	1.6 ± 0.2[c,d,e]	1.6 ± 0.3[c,d,e]
18:0	43.2 ± 1.2[b]	44.9 ± 2.9[b]	43.5 ± 1.9[b]	41.5 ± 2.2[b]	41.8 ± 0.7[b]
18:1	21.9 ± 1.4[b,c]	19.9 ± 0.9[b,c]	17.7 ± 3.3[d]	19.6 ± 1.6[b,d]	22.8 ± 0.7[c]
18:2n-6	0.6 ± 0.1[b]	0.4 ± 0.1[b]	0.4 ± 0.1[b]	0.7 ± 0.1[c]	0.6 ± 0.1[b]
18:3n-3	0.07 ± 0.03[b]	0.07 ± 0.02[b]	0.00 ± 0.00[b]	0.03 ± 0.01[b]	0.04 ± 0.02[b]
20:0	2.0 ± 0.1[b]	1.5 ± 0.1[c]	1.9 ± 0.2[b]	1.9 ± 0.1[b]	1.6 ± 0.3[b]
20:1	0.5 ± 0.1[b]	0.6 ± 0.1[b]	0.7 ± 0.2[b]	0.9 ± 0.1[b]	1.0 ± 0.1[c]
20:2n-6	0.2 ± 0.0[b]	0.2 ± 0.0[b]	0.08 ± 0.0[c]	0.2 ± 0.0[b]	0.2 ± 0.0[b]
20:3n-6	1.6 ± 0.4[b]	3.0 ± 0.6[c]	2.8 ± 0.5[c]	1.8 ± 0.3[b]	1.8 ± 0.2[b]
20:4n-6	18.8 ± 1.3[b,c]	17.2 ± 1.2[b,c]	15.2 ± 1.9[b,c]	19.6 ± 0.7[b]	20.4 ± 0.3[c,d]
22:6n-3	0.9 ± 0.1[b]	0.7 ± 0.[b]	0.6 ± 0.0[c]	0.9 ± 0.1[b]	0.9 ± 0.1[b]
Ratios					
20:3n-6/20:4n-6	0.1 ± 0.02[b]	0.2 ± 0.03[c]	0.2 ± 0.03[c]	0.1 ± 0.02[b]	0.1 ± 0.01[b]

[a]See legend to Table 2.

TABLE 5

The Fatty Acid Composition of PI in the Platelets of Subjects Before, During and After Consuming Borage Oil[a]

Fatty acids	Day 0	Day 22	Day 43	Day 64	Day 85
			(mol% of total)		
16:0	2.7 ± 0.2[b]	2.9 ± 0.1[b]	3.9 ± 0.7[b]	4.5 ± 0.7[b]	3.1 ± 0.2[b]
18:0	48.4 ± 1.6[b]	47.2 ± 1.1[b]	45.4 ± 1.6[b]	44.3 ± 2.4[b]	49.1 ± 1.4[b]
18:1	4.5 ± 0.5[b]	4.3 ± 0.4[b]	4.5 ± 0.6[b]	4.9 ± 0.6[b]	4.1 ± 1.2[b]
18:2n-6	0.4 ± 0.1[b]	0.4 ± 0.1[b]	0.5 ± 0.1[b]	0.7 ± 0.1[b]	0.5 ± 0.1[b]
20:0	0.6 ± 0.1[b]	0.5 ± 0.0[b]	0.6 ± 0.4[b]	0.6 ± 0.1[b]	0.4 ± 0.1[b]
20:2n-6	0.3 ± 0.0[b]	0.2 ± 0.0[b]	0.2 ± 0.1[b]	0.2 ± 0.0[b]	0.3 ± 0.2[b]
20:3n-6	0.5 ± 0.2[b]	1.3 ± 0.2[c]	1.7 ± 0.2[c]	0.4 ± 0.2[b]	0.7 ± 0.1[b]
20:4n-6	37.7 ± 2.3[b]	38.4 ± 1.8[b]	34.1 ± 2.6[b]	38.3 ± 1.1[b]	34.8 ± 4.1[b]
Ratios					
20:3n-6/20:4n-6	0.01 ± 0.004[b]	0.03 ± 0.005[b]	0.05 ± 0.008[c]	0.01 ± 0.009[b]	0.02 ± 0.005[b]

[a]See legend to Table 2.

TABLE 6

The Fatty Acid Composition of SPH in the Platelets of Subjects Before, During and After Consuming Borage Oil[a]

Fatty acids	Day 0	Day 22	Day 43	Day 64	Day 85
			(mol% of total)		
16:0	20.8 ± 0.9[b]	19.3 ± 0.9[b]	21.3 ± 0.9[b]	22.5 ± 1.0[b]	25.5 ± 0.7[c]
18:0	3.3 ± 0.5[b]	2.5 ± 0.2[b]	3.5 ± 0.5[b]	2.7 ± 0.5[b]	3.1 ± 0.4[b]
18:1	0.5 ± 0.1[b]	0.4 ± 0.1[b]	1.2 ± 0.4[c]	0.1 ± 0.1[b]	0.3 ± 0.1[b]
20:0	5.9 ± 0.7[b]	4.7 ± 0.8[c]	4.4 ± 0.5[c]	5.8 ± 1.0[b]	5.9 ± 0.8[b]
22:0	30.0 ± 1.0[b]	24.3 ± 1.3[c]	23.4 ± 0.8[c]	28.9 ± 0.7[b,d]	26.9 ± 0.6[b]
22:1	3.9 ± 0.9[b]	2.4 ± 0.4[c]	2.2 ± 0.4[c]	2.7 ± 0.7[b]	3.1 ± 0.6[b]
23:0	2.5 ± 0.2[b]	2.5 ± 0.1[b]	2.1 ± 0.1[c]	2.8 ± 0.2[b]	2.5 ± 0.1[b]
24:0	13.6 ± 1.5[b]	13.6 ± 1.3[b]	12.0 ± 1.3[b]	14.3 ± 2.0[c]	12.9 ± 1.5[b]
24:1n-9	16.2 ± 3.2[b]	27.6 ± 2.1[c]	24.7 ± 1.9[c]	17.1 ± 1.5[b]	16.8 ± 1.2[b]

[a]See legend to Table 2.

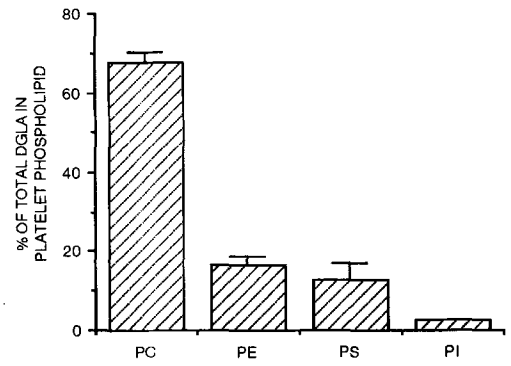

FIG. 2. Percentage contributions of individual platelet phospholipids to the total mass of DGLA in phospholipid at day 43. Values are means ± SEM (n = 6).

pholipid from plasma to platelet. In contrast, a significant rise in the AA level as found in human plasma phospholipid following the consumption of dietary evening primrose oil and/or borage oil containing GLA (17,37,38) may arise from a higher Δ5 desaturase activity in human liver.

The mass distribution of DGLA among the various phospholipids (Fig. 2) was in the order of PC >> PE > PS >> PI with no significant contribution from SPH. In contrast to the DGLA distribution, previous studies with dietary fish oils in humans have indicated that the order for the mass distribution of EPA was PE >> PC >> PS > PI (11) and for DHA was PE >> PC > PS >> PI (13).

Of considerable interest in this investigation was the marked alteration in the fatty acid composition of SPH: notably, the marked rise in NA upon borage oil ingestion without any detectable accumulation of DGLA or AA in this particular phospholipid. The rise in NA was accompanied by a significant decrease in 22:0 as well as 20:0, and 22:1 to a lesser extent without any significant decrease in 24:0. It is possible that the altered fatty acid compositions of the other phospholipids (PC, PE, PS, PI) induced by dietary borage oil may alter membrane fluidity which impacts upon the membrane-bound desaturase activity ultimately responsible for the formation of NA and/or enzymic reactions involved in SPH turnover. The possibility exists therefore that the altered fatty acid composition in sphingomyelin may play a role in maintaining membrane fluidity and function in response to the altered fatty acid composition of non-sphingomyelin phospholipids. Such may be imparted in the stabilizing effect of SPH species containing NA. It is also possible that the presence of 24:1n-9 and 22:1 in the borage oil could contribute to the rise of NA in SPH.

In conclusion, borage oil containing GLA was found to have a significant impact on the fatty acid composition of platelet membrane phospholipids with considerable differences being exhibited across the individual phospholipids. The increased DGLA levels and DGLA/AA ratios without an accompanying rise in AA (in the case of PC, PE, PS, PI), similar to the findings of other studies using evening primrose oil (17-19), and the marked rise in NA in SPH were of particular interest. The biochemical factors regulating these compositional shifts and the potential physiological significance of such alterations remain to be investigated.

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The Effect of Short-Term Lipid Infusion on Liver Function and Biliary Secretion in Rats

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This study was undertaken to determine the effect of various lipid emulsions on the hepato-biliary system in rats. Rats were randomly divided into six groups and infused continuously for 48 hr with either long-chain triglycerides (LCT), medium-chain triglycerides (MCT) or a mixture of MCT and LCT. One group infused with physiological saline solution served as controls. Throughout this period the rats received a fat free diet *ad libitum*. During the last hour of lipid infusion bile was collected for determination of bile flow and composition. Subsequently, the rats were sacrificed and the morphology and lipid content of the liver determined. Only LCT lipid emulsions induced morphological changes and increased liver cholesterol content. In two rats infused with radiolabeled LCT, no labeled cholesterol was found in the liver, indicating that the excess hepatic cholesterol level may originate from enhanced cholesterol mobilization to the liver. Biliary cholesterol and phospholipid concentrations in LCT-treated rats were also elevated, as was the lithogenic index, whereas the other emulsions had no such effects. None of the emulsions affected the plasma liver function tests or bile flow. We therefore conclude that the lithogenicity of the bile in rats is directly related to the lipid components of the total parenteral nutrition and the type of triglyceride infused.
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Total parenteral nutrition (TPN) may result in the impairment of liver functions and the development of gallstones (1-5). The role of the lipid emulsion contained in the TPN in these complications is a controversial issue (6-10). One study in humans has indicated a possible involvement of the fat emulsion in causing abnormal liver function (6), but other reports showed that isocaloric substitution of glucose with fat emulsion did not alter the normal values of liver enzymes (7-9). Capron *et al.* (10) suggested that intestinal bacterial overgrowth with the production of endotoxin or hepatotoxic bile acids might explain some of the liver dysfunctions associated with TPN. Other investigators suggested that this complication might be prevented by oral supplements (11).

An increased incidence of cholelithiasis associated in patients with TPN has also been noted (2). Gimmon *et al.* (12) found that continuous administration of TPN solutions supplemented with long-chain triglycerides (LCT) to rats caused an increase in the lithogenicity of their bile independent of the route of administration, whether oral or intravenous. A recent study in rats (13) found that only TPN supplemented by LCT lipid emulsion of polyunsaturated fatty acids induced a significant increase in biliary cholesterol and phospholipid levels. In contrast, Innis (14), as well as Heyman *et al.* (15), reported that lipid-containing TPN reduced bile flow in rats, with no changes in bile composition. Other reports (16,17) have claimed that fat infusion to rats resulted in a decreased molar concentration of cholesterol and bile lithogenicity. The reason for these discrepancies is not clear.

In an attempt to define the role of lipid emulsions on liver function and bile lithogenicity, we studied the effect of short-term infusion of various lipid emulsions on liver function, bile composition and lithogenicity in rats which were fed with a fat-free oral diet. The rat was selected as a model since it has already been investigated in many TPN studies, although it lacks a gallbladder. This study indicates that infusion of the commonly used emulsion with long-chain triglycerides results in an increase in hepatic cholesterol content and in biliary cholesterol and phospholipids, whereas no such effects were observed in rats infused with medium-chain triglycerides (MCT) or mixtures of MCT and LCT.

MATERIALS AND METHODS

Experimental model. Male Sprague-Dawley rats (Mollegaard Ltd., Skensved, Denmark) of 230-270 g were housed in individual metabolic cages, at a controlled temperature (22 ± 3°C), light cycle (18.00-06.00 hr darkness) and relative humidity (40-60%). They were allowed water and standard rat pellets (R3 Ewos AB, Sodertalje, Sweden) *ad libitum* for a week before the experiment. A permanent central venous catheter was positioned under general anesthesia (Hypnorm, 0.5 mL/kg *i.m.*) in the lower part of the superior *vena cava* after its introduction in the right internal jugular vein. The catheter was then tunneled subcutaneously to the back of the rat and threaded through a metallic spiral connected to a ball bearing at the top of the metabolic cage. This permitted free movement of the rats, which were fitted with a harness (18). Infusions were started on the seventh day after the operation. Animals were checked daily for general health and body weight.

Study design. A total of 65 animals were randomly assigned to six experimental groups. All animals received infusions at a rate of 35 mL/kg/day. Group A was infused with saline and served as controls. Groups B-F were infused with lipid emulsions all containing 20% TG emulsified by 1.2% egg phosphatidylcholine (PC) and 2.5% glycerol in serile water. The fatty acid composition of the various emulsions is given elsewhere (19). Group B received a LCT lipid emulsion containing long-chain

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CSI, cholesterol saturation index; LCT, long-chain triglycerides; LDH, lactate dehydrogenase; LLL, long, long, long-chain fatty acyl triacylglycerols; MCT, medium-chain triglycerides; MLL, medium, long, long-chain fatty acyl triacylglycerols; MML, medium, medium, long-chain fatty acyl triacylglycerols; MMM, medium, medium, medium-chain fatty acyl triacylglycerols; PC, phosphatidylcholine; TG, triglycerides or triacylglycerols; TPN, total parenteral nutrition.

triglycerides (TG) (C_{14}-C_{24}) (Endolipid 20%, B. Braun, Melsungen, Germany). Group C was infused with LCT lipid emulsion (Intralipid 20%, Kabi, Sweden) containing long-chain TG (C_{14}-C_{24}). Group D received MCT/LCT lipid emulsion (Lipofundin MCT/LCT 20%, B. Braun, Melsungen, Germany), containing a mixture of equal amounts (w/w) of LCT (C_{14}-C_{24}) and MCT (C_6-C_{12}). Group E was infused with a "structured lipid" emulsion (MCT + LCT 20% Kabi) containing 75 mole% of MML + MLL and 25 mole% of MMM + LLL. Group F was infused with MCT 20% lipid emulsion (Kabi, Sweden) containing medium-chain fatty acids (C_6-C_{12}).

In addition, two rats underwent the same protocol and were infused with radiolabeled [^{14}C]LCT emulsion prepared with 2 mL of Intralipid 20% containing (4 μCi/mL) glycerol tri-[1-^{14}C]oleate per 100 mL Intralipid. The administered activity was approximately 2.75 μCi/kg body weight/day.

During the infusion period for 48 hr all the rats had free access to water and semisynthetic fat-free pwoder diet containing 20% casein supplemented with 0.3% L-methionine, minerals and trace elements (20) (Table 1). After 48 hr of infusion the rats were anesthetized with Hypnorm 0.5 mL/kg and Ketamin HCl (*i.m.*), a midline laparotomy was performed, the common bile duct was cannulated with a polyethylene catheter (PE 10), and the abdomen was closed. During the last hour of infusion, bile was collected while the rats remained anaesthetized and the infusions continued at the same rate. The effect of anesthesia and bile salt reabsorption on bile composition were minimized by limiting this procedure to one hour. The rate of bile flow was measured with a stopwatch. After completing collection of bile for one hour, the bile was frozen at −70°C for a maximum of four weeks before determination of cholesterol, phospholipids and bile-salts concentrations. Four rats (one of each of the following groups: Endolipid, Intralipid, structured and MCT) were withdrawn from the study due to technical problems in collecting bile.

Liver function. At the end of the study the animals were killed by cardiac aspiration and aliquots of blood were taken for determination of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). These liver function tests were performed by standard photometric techniques (21).

The liver was excised and weighed. Approximately 1 g of liver was placed in a 10% buffered formaldehyde solution for histologic examination. The rest of the liver was frozen at −70°C for later total cholesterol determination.

Bile analysis. Bile acid concentration was determined enzymatically (22), cholesterol was determined by the method of Abell *et al.* (23), and phospholipid phosphorus was measured as described by Bartlett (24). The cholesterol saturation index (CSI) was calculated using the critical tables of Carey (25).

Liver structure. For light microscopy studies, portions of fresh liver obtained at the time of death were fixed in formaldehyde 10% and stained with hematoxylin-eosin and oil-red. Quantitative evaluation of fatty liver infiltration was estimated on slides under light microscopy as percentage of hepatocytoplasm infiltrated with fat globules as described by Leevy (26).

Hepatic cholesterol and radioactive cholesterol in liver and bile. Liver samples of 1 g were homogenized with 4 mL of saline using an Ultra-Turrax (Janke and Kunkel Ika-Werk, Staufen, Germany). The homogenates were then extracted with chloroform/methanol according to Bligh and Dyer (27), and cholesterol was determined by the method of Abell *et al.* (23). For determination of radiolabeled cholesterol, the isolated lipids were first passed through a silicic acid column. Triglycerides and cholesteryl esters were eluted with hexane/diethyl ether (9:1, v/v); cholesterol, fatty acids and diglycerides were eluted with chloroform. To isolate cholesterol, the chloroform eluate was subsequently subjected to alkaline hydrolysis. Cholesterol and fatty acids were extracted into hexane and separated by thin-layer chromatography, employing hexane/diethyl ether/methanol/acetic acid (90:20:2:3, by vol.) as solvent. The zone corresponding to cholesterol was scraped into a scintillation vial and counted using a Kontron Scintillation Spectrometer (Hemile, Switzerland). The amount of cholesterol present in the sample was determined according to Abell *et al.* (23), yielding information on cholesterol recovery.

To isolate radioactive biliary cholesterol, bile lipids were extracted with chloroform/methanol (27). One half of this sample was used for thin-layer chromatography and counted using a Kontron Scintillation Spectrometer, the other half was quantitatively analyzed for cholesterol as previously described.

Statistical analysis. Significance of differences were tested using ANOVA with multiple comparison (Dunnet's tests). All data represent average values ± SD.

TABLE 1

Composition of Fat-Free Diet (g/kg) Supplied During the Infusion Period

Vitamin free casein	200
L-methionine	3
Sucrose	250
Wheat starch	250
Glucose	223.7
Cellulose powder	10
Vitamin mix I[a]	5
Vitamin mix II[b]	5
Mineral mix[c]	53.3

[a] Per 5 g vitamin mix I, choline chloride, 2.0 g; and sucrose, 3.0 g.

[b] Per 5 g vitamin mix II, thiamine mononitrate, 5 mg; riboflavin, 8 mg; pyridoxine HCl, 5 mg; niacinamide, 40 mg; Ca-pantothenate, 40 mg; *p*-aminobenzoic acid, 2 mg; biotin, 4 mg; folic acid, 2 mg; vitamin B12, 30 μg; inositol, 100 mg; and sucrose to 5 g.

[c] Per 53.3 g mineral mix, NaCl, 6.97 g; KH_2PO_4, 19.46 g; $MgSO_4 \cdot 7H_2O$, 5.87 g; $CaCO_3$, 19.08 g; $FeSO_4 \cdot 7H_2O$, 1.35 g; $MnSO_4 \cdot H_2O$, 0.20 g; $ZnSO_4 \cdot 7H_2O$, 82 mg; $CuSO_4 \cdot 5H_2O$, 24 mg; $CoCl_2 \cdot 6H_2O$, 1 mg; $Na_2SO_4 \cdot 10H_2O$ 1/1000 (with sucrose), 234 mg; and KI, 39 mg.

RESULTS

This paper presents the effect of short-term infusion of various lipid emulsions on liver morphology and on the lipid composition of the heptobiliary system. Five groups of rats were infused with different lipid emulsions and compared with a control group that was infused with a saline solution. All the rats were fed with a fat-free oral diet *ad libitum* (Table 1), while being infused with the various solutions. It is of note that the control group, which was deprived of any dietary lipids while receiving

saline infusion, consumed approximately 15% more food than all the other groups (Table 2) to compensate for the lack of calories; over the 48-hr experiment this group showed a weight gain similar to that of the other rats (Table 2). There were no significant differences in weight between the various groups either before or after the 48 hr of infusion (Table 2).

None of the infusion regimens affected liver weight or enzymes (Table 3). Morphologic studies of the liver with light microscopy revealed no cell necrosis or granulomatos changes in any of the groups. Fatty infiltration was apparent only in the liver of rats infused with LCT emulsions (Endolipid™ and Intralipid®); no such effect was observed in rats infused with either MCT or mixtures of MCT and LCT.

The plasma bilirubin levels were not altered by infusion of either MCT or mixtures of MCT and LCT (Table 3). Similar bilirubin levels were observed in rats treated with the Endolipid (LCT) emulsion. In contrast, there was significant hyperbilirubinemia in those rats infused with Intralipid (Table 3), in apparent contradiction with the findings of Gimmon *et al.* (12), who observed no such effect.

Another significant effect of both LCT emulsions (Intralipid and Endolipid) was the increase of hepatic cholesterol level (Table 3). The level of hepatic cholesterol in the LCT-treated rats was significantly higher (by 30–50%) than in the control group ($P < 0.05$; Table 3). Infusion of structured MCT + LCT emulsion, MCT or Lipofundin MCT/LCT did not affect hepatic cholesterol content (Table 3).

None of the emulsions affected the rate of bile secretion (Table 4). However, infusion of LCT emulsions (either Intralipid or Endolipid) caused a significant elevation (by approximately 70%; $P < 0.01$) of biliary cholesterol and a somewhat smaller increase (approximately 50%; $P < 0.05$) in biliary phospholipid concentrations (Table 4). Following these LCT infusions, the CSI of the bile of all 20 rats (both Intralipid and Endolipid groups taken together) was significantly higher ($P < 0.05$) than after infusion of saline to the control group (Table 4). Neither MCT nor MCT/LCT, nor the "structured" lipid emulsion had any such effect on bile lipids (Table 4). It is of interest that although the average increase of hepatic cholesterol concentration was quantitatively similar to that observed for

TABLE 2

Weight and Food Intake of the Rats[a]

	Control	LCT		MCT/LCT		MCT
	Saline	Endolipid	Intralipid	Lipofundin	Structured	MCT
Group	A	B	C	D	E	F
No. of rats	(11)	(10)	(10)	(10)	(9)	(9)
Weight A[a] (g)	287.5 ± 16.2	297.6 ± 15.2	293.9 ± 8.2	297.4 ± 15.0	295.4 ± 16.0	279.7 ± 16.7
Weight B[a] (g)	292.4 ± 15.3	304.2 ± 17.9	301.4 ± 9.9	305.1 ± 15.3	296.1 ± 19.0	282.7 ± 14.2
Food intake (g)	46.4 ± 5.8	41.7 ± 5.0	39.9 ± 5.5	39.1 ± 7.4	40.6 ± 1.8	39.1 ± 2.5

[a]The animals were weighed prior to (weight A) and immediately after (weight B) the 48 hr of infusion. Food intake is based on subtraction of the residudal food from the fat-free diet given to the rats during the infusion period.

TABLE 3

Liver Function Tests, Relative Liver Weight (RLW) and Hepatic Cholesterol Levels in Rats Infused with Lipid Emulsions

	Control	LCT		MCT/LCT		MCT
	Saline	Endolipid	Intralipid	Lipofundin	Structured	MCT
Groups	A	B	C	D	E	F
No. of rats	(11)	(10)	(10)	(10)	(9)	(9)
AST[a] (ukat/L)	4.9 ± 1.2	4.4 ± 1.5	6.1 ± 2.1	4.7 ± 1.1	4.2 ± 0.9	4.4 ± 1.2
ALT[a] (ukat/L)	1.6 ± 0.5	1.6 ± 0.9	1.8 ± 0.9	1.3 ± 0.4	1.5 ± 0.5	1.9 ± 0.3
LDH[a] (ukat/L)	45.6 ± 17.3	37.9 ± 10.4	51.8 ± 19.2	45.8 ± 14.2	39.0 ± 14.6	40.3 ± 12.9
ALP[a] (ukat/L)	6.9 ± 5.8	4.6 ± 0.4	4.4 ± 0.9	4.5 ± 0.3	4.8 ± 0.9	4.7 ± 0.7
Bilirubin (mg/dL)	1.7 ± 0.6	0.9 ± 0.9	3.4 ± 1.2[b]	2.2 ± 0.9	1.7 ± 0.9	1.7 ± 0.6
Liver weight (g)	12.7 ± 1.2	13.3 ± 1.0	13.3 ± 1.1	12.8 ± 0.7	13.7 ± 0.8	11.6 ± 1.3
RLW (g/kg)	44.3 ± 2.9	44.7 ± 3.3	44.8 ± 3.1	43.4 ± 1.7	45.9 ± 2.0	41.7 ± 3.5
Cholesterol (mg/g Liver)	1.7 ± 0.9	2.6 ± 1.2[c]	2.2 ± 0.5[c]	1.5 ± 0.4	2.2 ± 0.7	1.6 ± 0.8

[a]Abbreviations used are: AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; and ALP, alkaline phosphatase.

[b]Significance of the difference between the bilirubin level observed in group C, in comparison to group A, is smaller than P = 0.001. All the other differences in values compared with group A were insignificant.

[c]Hepatic cholesterol level in the LCT-treated rats (groups B ± C) is significantly different ($P < 0.05$) than in the control group (A).

TABLE 4

Bile Flow and Bile Composition in Rats Infused with Lipid Emulsions

	Control	LCT		MCT/LCT		MCT
	Saline	Endolipid	Intralipid	Lipofundin	Structured	MCT
Groups	A	B	C	D	E	F
No. of rats	(11)	(10)	(10)	(10)	(9)	(9)
Flow (mL/hr)	0.91 ± 0.30	1.05 ± 0.34	0.89 ± 0.15	1.00 ± 0.22	0.98 ± 0.09	0.99 ± 0.11
Cholesterol (mM)	0.16 ± 0.07	0.28 ± 0.13[a]	0.28 ± 0.12[a]	0.21 ± 0.04	0.19 ± 0.05	0.22 ± 0.04
Phospholipid (mM)	0.88 ± 0.34	1.33 ± 0.60[b]	1.35 ± 0.37[b]	1.01 ± 0.27	0.98 ± 0.24	1.12 ± 0.20
Bile salts (mM)	16.6 ± 6.3	16.7 ± 7.5	20.2 ± 5.9	17.9 ± 3.5	16.9 ± 4.1	21.4 ± 5.1
CSI[c]	0.64 ± 0.38	0.82 ± 0.29[d]	0.72 ± 0.41[d]	0.63 ± 0.15	0.63 ± 0.15	0.57 ± 0.08

[a]Cholesterol levels were significantly higher in groups B or C in comparison to group A ($P < 0.01$).

[b]The biliary phospholipid levels in groups B or C were significantly higher ($P < 0.05$) than in group A.

[c]The cholesterol saturation index (CSI) was calculated on the basis of Carey's tables (ref. 25).

[d]The CSI in groups B and C were significantly higher than in group A ($P < 0.05$) only when taken together.

biliary cholesterol, no correlation (R = 0.5; P = 0.46) was found between the levels of hepatic and biliary cholesterol in the individual rats.

In an attempt to clarify the mechanism responsible for the effect of LCT lipid emulsions on bile composition, we infused radiolabeled Intralipid (2.75 μCi/kg/day) in two rats following the same protocol and determined the radioactivity of cholesterol in their liver and bile after completion of the infusions. No radioactive cholesterol was found in these samples although large quantities of cholesterol were detected, with efficient recovery of cholesterol in the experiment (greater than 90%), and in spite of the infusion-induced elevation of hepatic and bile cholesterol levels.

DISCUSSION

The effect of lipid infusion on bile composition in rats is a controversial and problematic issue (12–17). The present study was designed so that the rats were fed a fat-free diet while being infused with various lipid emulsions. This protocol minimized the potential effects of starvation and of non-lipid components of TPN on hepatic and bile composition and enabled us to evaluate the net effect of lipid.

The results obtained in this study show that short-term infusion of long chain triglycerides caused fatty infiltration of the liver and resulted in a significant increase of hepatic cholesterol as well as in elevation of biliary cholesterol and phospholipids. No such effects were observed in rats infused with either MCT or mixtures of MCT and LCT. These results are consistent with the finding (28–30) that the infused MCT-containing emulsions are oxidized more rapidly and are therefore accumulated to a lesser extent in the liver than are LCT emulsions. These results concur with those described by several other groups (28–30), but contradict a recent report (31) that MCT induced greater fat infiltration of the liver than did LCT lipid emulsion.

Since the infused emulsions do not contain cholesterol, the excess cholesterol in the liver and bile observed following infusion of LCT may be due either to cholesterol mobilization by emulsion remnants from extrahepatic sources to the liver or increased *de novo* synthesis of cholesterol in the liver (19,32). The latter possibility is unlikely in light of the absence of radiolabeled cholesterol in the liver or bile of rats infused with [^{14}C]LCT, in spite of the infusion-induced elevation of hepatic and biliary cholesterol level. It would appear that the excess hepatic and biliary cholesterol following lipid infusion resulted mainly from mobilization of cholesterol to the liver from extrahepatic tissues, commonly denoted as "reverse cholesterol transport" (19). This conclusion accords with previous reports on the relative efficiency of LCT and MCT remnants in mobilizing cholesterol to the liver (33); MCT and its mixtures with LCT are more efficiently metabolized in the liver (28,29), but have a lower ability to mobilize cholesterol to the liver than do LCT (19,33–35). Indeed, MCT-containing emulsions did not cause a significant increase of hepatic cholesterol in rats, lending further support to our conclusions.

A point of interest is the lack of correlation between the increase in hepatic and biliary cholesterol in individual rats. This may result from the complex effect of other factors on bile composition (35,36). More specifically, biliary cholesterol secretion is regulated by numerous factors, including synthesis, esterification of cholesterol in the liver and the existence of different intracellular pools as suggested by Nervi *et al.* (37). Therefore, because of this complexity, individual differences in the metabolic fate of excess hepatic cholesterol may obscure any correlation between hepatic and biliary cholesterol levels.

Hepatic phospholipids newly synthesized from long-chain fatty acids have been reported to make only a small contribution to the total biliary output (36,38). The elevation of biliary phospholipids, observed in this study following LCT infusion, is therefore likely to originate from an extrahepatic source.

The effect of lipid infusion on bile lithogenicity as expressed by the CSI is rather complex. Neither MCT nor mixtures of MCT and LCT affected the levels of any of the biliary lipids. Thus, neither of them altered the CSI (25). The LCT induced elevation of cholesterol could have been expected to increase the CSI, whereas the opposite could have been expected from the increase in biliary phospholipids. A slight and insignificant increase was observed in the CSI of the bile of rats infused with either Intralipid or Endolipid when compared to the control group. However, when the LCT lipid emulsions (Intralipid

and Endolipid groups) were taken together a significant increase in the CSI of the bile was observed. This is despite the increase in biliary phospholipids, which in itself should have reduced the CSI (25).

In conclusion, the results reported here show that short-term infusion of LCT emulsions (but not of MCT, nor of various mixtures of LCT and MCT) in the rat cause a significant increase of both hepatic and biliary cholesterol, probably *via* a cholesterol mobilization mechanism. The effects of LCT infusion on bile composition observed in the present study in rats are similar to those found in our previous study carried out on human subjects (39). However, in the human, in contrast to the rat, MCT/LCT lipid emulsions caused an even more pronounced elevation of both biliary cholesterol and phospholipid (39). We suggest that the difference in effectiveness of MCT/LCT infusion on biliary lipids observed in humans and in rats may be ascribed to a difference between the species (40). The data presented here may contribute to our understanding of the role of lipids in the hepatobiliary abnormalities associated with total parenteral nutrition.

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Effect of Dietary Fish Oil on the Rate of Very Low Density Lipoprotein Triacylglycerol Formation and on the Metabolism of Chylomicrons

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The mechanism by which ω3 fatty acids lower plasma triacylglycerol levels was investigated. Rats were fed fish oil, olive oil (10% fat by weight) or a nonpurified diet (4% fat by weight) for 15 days. Lipoprotein lipase was inhibited by intra-arterial administration of Triton WR 1339 to estimate hepatic triacylglycerol output. Rats fed the olive oil diet showed a higher rate of triacylglycerol formation than rats fed the ω3 fatty acid diet or the low-fat diet. All three groups showed identical rates of removal from plasma of intraarterially administered artificial chylomicrons that had simultaneously been labeled with cholesteryl [1-^{14}C]oleate and [9,10(n)-^{3}H]triolein. Liver radioactivity and total fat content were lowest in rats fed the fish oil diet, indicating that ω3 fatty acids were preferentially metabolized in liver. Chylomicrons obtained from donor rats fed either fish oil containing [^{14}C]cholesterol or olive oil containing [^{3}H]cholesterol were removed at similar rates when infused together intraarterially into recipient animals. A slower formation of plasma very low density lipoprotein triacylglycerols in rats fed fish oil is probably due to a faster rate of oxidation of the fatty acid chains in the liver resulting in decreased plasma triacylglycerol concentrations.
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The role that dietary ω3 eicosapentaenoic and docosahexaenoic acids of marine oils play in the prevention of coronary heart disease has been studied extensively (1,2). Fish oils are known to lower plasma triacylglycerol (TG) levels and very low density lipoprotein (VLDL) levels both in humans (3-5) and in experimental animals (6), although the mechanisms that are involved are not clearly understood.

Fish oil has been shown to retard *in vivo* hepatic production of VLDL triacylglycerol in humans (5,7-10) and in roosters (11), as well as in perfused liver (12,13) and in cultured hepatocytes (14-16). Less evidence is available in support of the enhancing effect of fish oil on the removal of plasma VLDL (7,9). Fish oil can also influence chylomicron metabolism, although similar intestinal absorption rates for eicosapentaenoic acid and oleic acid have been reported (17). However, the results of chylomicron metabolism studies are not entirely conclusive. When analyzed between 25 and 90 min, the disappearance rate of eicosapentaenoic acid enriched chylomicrons, that had been infused in the bloodstream of rats, was slower than that of oleic acid enriched particles. At 10 min, no difference in turnover rates was observed between the two types of chylomicrons (18). Chylomicron clearance rates in perfused rat hearts were similar for particles made from ω3 fatty acid with those made from oleic acid (19).

We set out to determine whether the fats affected metabolism because the particles differed in fatty acid composition or because the dietary fats by themselves modified lipoprotein lipase activity and the splanchnic organ receptors for chylomicron particles. In our study, native chylomicrons from animals fed fish oil and [^{14}C]cholesterol, or olive oil and [^{3}H]cholesterol, were injected into rats fed a nonpurified low-fat diet. Also, the plasma disappearance rates of intra-arterially infused artificial chylomicrons simultaneously labeled with cholesteryl [1-^{14}C]oleate and [9,10(n)-^{3}H]triolein were compared in rats fed fish oil, olive oil and low fat. Triacylglycerol mass and radioactivity were measured in the liver and spleen of the animals at the end of the artificial chylomicron infusion experiment. Finally, the effect of the different diets on hepatic secretion of triacylglycerol and on the metabolism of chylomicrons was examined after inhibition of the enzyme lipoprotein lipase by Triton WR 1339.

MATERIALS AND METHODS

Experimental protocol. Adult male Wistar rats, weighing approximately 230 g, were divided into three experimental groups of 10 animals each. The animals were fed for 15 days (% by weight) 10% fish oil, 10% olive oil, or a nonpurified diet containing 4% fat (Control, provided by Novilab, São Paulo, Brazil). Both fat enriched diets contained (% by weight): fat (10%), casein (10%), cellulose (3.5%), saline mixture (3.5%), vitamin supplement (1.0%), choline bitartrate (0.2%), D,L-methionine (0.3%), and starch (71.5%).

Body weights (mean ± SD) of rats in experimental groups (initial and final, respectively) were: controls (195 ± 19 and 244 ± 33), fish oil (250 ± 22 and 264 ± 28), and olive oil (235 ± 58 and 268 ± 38).

Commercially available fish oil (MaxEPA, Seven Seas Ltd., Marfleet, England) contained 18.6% eicosapentaenoic acid, 12.1% docosahexaenoic acid, 15.2% oleic acid, 22.95% saturated fatty acids and 31.3% unidentified unsaturated fatty acids. Olive oil was composed of 77.5% oleic, 7.6% linoleic, 2.3% stearic, 10% palmitic, and 0.6% unidentified fatty acids. After fasting overnight, rats were treated with Triton WR 1339 (Tyloxapol, Sigma Chemical Co., St. Louis, MO; 600 mg/kg body weight), which was administered through an intra-arterial catheter, to evaluate hepatic production of triacylglycerols (20).

Rats were infused intra-arterially with chylomicrons that had been simultaneously labeled with cholesteryl [1-^{14}C]oleate and [9,10(nn)-^{3}H]triolein (21,22) (NEN Research Products, Boston, MA). To follow the effect of various diets on the rate of chylomicron removal from plasma, a chylomicron-like emulsion was used which consisted of (% by weight) 2% cholesterol, 23% phosphatidyl-

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; FCR, fractional catabolic rate; HDL, high density lipoproteins; LDL, low density lipoproteins; TG, triacylglycerol; VLDL, very low density lipoproteins.

choline (Lipid Products, Surrey, U.K.), 6% cholesteryl oleate and 69% triolein (NuChek Prep, Inc., Elysian, MN). Lipids were dispensed from stock solutions into vials and radioactive materials were added. After evaporation of solvents under a nitrogen stream, the vials were placed overnight in a vacuum desiccator at 4°C to remove residual solvent. Lipids were emulsified by sonication in 8 mL of 2.785 M NaCl solution (density = 1.100 g/mL), at approximately 55°C using a Branson Cell Disruptor (Branson Ultrasonics Corp., Danbury, CT) and a 1-cm probe at a continuous output of 70–80 W. Crude emulsions were purified by ultracentrifugation using an SW-41 rotor (Beckman ultracentrifuge, model L7-55, Beckman Instruments, Palo Alto, CA) at 12,000 rpm (density gradient 1.100–1.006) at 20°C for 15 min. After the first run, the coarse lipid that floated to the top was removed by aspiration and replaced with a corresponding volume of fresh 1.006 g/mL solution. The chylomicron-like emulsion was again ultracentrifuged at 36,000 rpm for 25 min. The emulsion particles floating to the top were then aspirated and used for the analyses and metabolic studies. The lipid composition of the emulsions was determined by standard procedures. Cholesterol was analyzed by the enzymatic Chod-Pap method (Boehringer Mannheim, Mannheim, Germany). Triacylglycerols were measured using the enzymatic Biodiagnostica kit (São Paulo, Brazil). Phospholipids were assayed by the Bartlett method (23). Chylomicron composition was (% by weight): cholesterol, 1.9%; phospholipids, 10.4%; cholesteryl ester, 11.2%; and triacylglycerol 76.5%.

Approximately 159 μg of chylomicron triacylglycerol was infused into each rat together with cholesteryl (1-^{14}C)oleate (0.34 μCi) and [9,10(n)-^{3}H]triolein (1.17 μCi). Blood samples were drawn sequentially, and 10 min later the liver and spleen were rapidly removed and analyzed for triacylglycerol total fat and radioactivity levels.

Native chylomicrons drawn from the pooled intestinal lymph of donor rats that had been fed fish oil and [^{14}C]-cholesterol, or olive oil and [^{3}H]cholesterol, were pooled and pulse infused intra-arterially into recipient rats fed the non-purified diet to measure the rates of disappearance of both chylomicron species from plasma. Each rat received 8.45 mg of chylomicron triacylglycerols from each dietary source containing ^{14}C (0.43 μCi) and ^{3}H (0.93 μCi). In these experiments adult male Wistar rats were subjected to intestinal lymph duct catheterization under pentobarbital anesthesia (5 mg/100 g of body weight) and restrained in Bollman-type metabolic cages. A single dose of fish oil or olive oil containing radioactive cholesterol was administered to each rat by gavage 24 hr later. The intestinal lymph was collected on ice in 0.01% sodium ethylenediaminetetraacetic acid (EDTA) over a period of 24 hr while the animals drank 5% glucose solution in saline *ad libitum*. For native chylomicron separation, the lymph was submitted to ultracentrifugation at 24,500 rpm at 20°C (density = 1.006 g/mL) using an SW 41 rotor (Beckman ultracentrifuge model L3-50, Beckman Instruments). Chylomicrons were harvested with a Pasteur pipette and were used within 24 hr.

Plasma analyses. Blood was drawn after carotid catheterization. In the Triton WR 1339 experiment, blood was sequentially drawn over a 90 min period and triacylglycerols were measured enzymatically (Biodiagnostica). Triacylglycerols secreted by the liver were calculated by linear regression of the plasma triacylglycerol concentration data on the ordinate as related to time (on the abscissa) according to the equation $y = a + bx$, where b is the angular coefficient of the minimum square straight line, expressed in mg/100 mL/min.

In the artificial chylomicron infusion experiments, blood was drawn at 2, 4, 6, 8 and 10 min and plasma (100 μL) was extracted with chloroform/methanol (2:1, v/v). Radioactivity in the triacylglycerol and cholesteryl ester fractions was measured as previously described (21). In the natural chylomicron infusion experiment, ^{14}C and ^{3}H radioactivities were measured in blood samples drawn as described above; plasma aliquots were counted using a toluene-phosphor solution and a Beckman LS-100 beta scintillation counter (Beckman Instruments, Inc.) (24).

The fractional removal rate for each isotope in the plasma was computed from a monoexponential curve fitting after plotting the plasma radioactivity log values (on the ordinate) against time in minutes (on the abscissa). A monoexponential least square line as drawn ($y = a \cdot e^{bx}$), where b represents the angular coefficient which is the fractional removal rate expressed in minutes (21).

Tissue analyses. Aliquots of liver and spleen (1g) were extracted with 30 mL of a solution of chloroform/methanol (2:1, v/v). After filtration and addition of water (7 mL), the upper water phase was discarded. A solution of chloroform/methanol/water (3:48:47, v/v/v; 4 mL) was then added, and the water phase discarded. The solvents were evaporated under a stream of nitrogen. The extracted lipids were purified by thin-layer chromatography (TLC) on silica gel using the solvent system hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The cholesteryl ester and triacylglycerol bands were identified in an iodine chamber and eluted with diethyl ether. Radioactivity was determined in a toluene phosphor scintillation solution. An aliquot of the extracted tissue fat was used for the gravimetric measurement of total fat and triacylglycerols using the enzymatic kit (Biodiagnostica; Clinical Chem. Industry, São Paulo, Brazil).

RESULTS

Triacylglycerol and cholesterol concentrations in plasma after 15 days on each diet are shown in Table 1. Although plasma lipids were measured in the fasting state, the plasma triacylglycerol level was much higher in the olive oil group than in the fish oil and control groups. Plasma cholesterol concentration was slightly, but significantly, higher in the fish oil fed rats than in the olive oil fed and control animals. Thus fish oil reduced plasma triacylglycerol concentration, but increased the plasma cholesterol level, in agreement with results reported by others for humans and animals (25,26).

Figure 1 shows the increase in plasma triacylglycerol level with time after intraarterial administration of Triton WR 1339. Formation of liver triacylglycerol was significantly lower in controls and in fish oil fed rats than in olive oil fed animals. Fractional removal rates of the artificial chylomicrons are shown in Table 2. The plasma fractional catabolic rate (FCR) of [9,10(n)-^{3}H]triolein measures the triacylglycerol lipolysis and the particle removal rates altogether, while the FCR of cholesteryl [1-^{14}C]oleate measures the particle removal rate only. The FCR of both radioactive moieties did not differ among the three ex-

TABLE 1

Plasma Levels of Triacylglycerols and Cholesterol in Rats After 15 Days of Dietary Treatment[a]

Experimental groups	Triacylglycerols	Cholesterol
Controls (n = 7)	94.3 ± 19.6	56.3 ± 9.4
Fish oil (n = 9)	98.3 ± 34.3	75.2 ± 14.3
Olive oil (n = 7)	170.4 ± 48.1	62.7 ± 11.8

[a]Mean mg/dL ± SD. Statistical comparisons between groups by Student's t-test was as follows:

Controls × fish oil	NS	$p < 0.005$
Controls × olive oil	$p < 0.005$	NS
Fish oil × olive oil	$p < 0.005$	$p < 0.05$

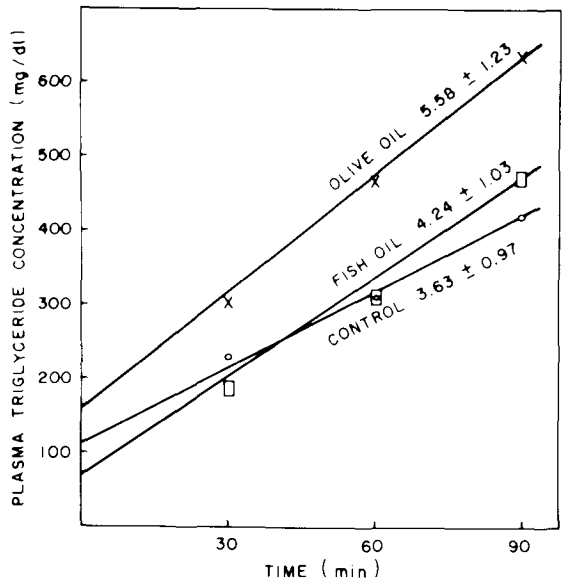

FIG. 1. Plasma triacylglycerol secretion rate. Variation of plasma triacylglycerol concentration after intra-arterial pulse infusion of Triton WR-1339. Control group: circles (n = 7); olive oil group: crosses (n =); fish oil group: squares (n = 9). Statistical comparisons: controls × olive oil ($p < 0.01$); control × fish oil (p = not significant); olive oil × fish oil ($p < 0.05$). For calculation of secretion rates, see text.

TABLE 2

Fractional Catabolic Rates of Artificial Chylomicrons Doubly Labeled with Cholesteryl [1-^{14}C] oleate [^{14}C-CE] and [9,10(n)-^{3}H] triolein [^{3}H TG] Pulse Infused Intra-Arterially[a]

Experimental groups	^{14}C-CE	^{3}H-TG
Controls (n = 10)	0.23 ± 0.09	0.36 ± 0.14
Fish oil (n = 10)	0.21 ± 0.04	0.34 ± 0.11
Olive oil (n = 10)	0.21 ± 0.04	0.28 ± 0.07

[a]Mean min^{-1} ± SD. Differences between experimental groups by Student's t-test were not significant.

perimental dietary groups. Prolonged periods of feeding the diets did not interfere with the rate of metabolism of the artificial chylomicrons in plasma.

After the simultaneous intra-arterial injection into control rats, the fractional removal rate of the native chylomicrons containing radioactive cholesterol, labeled either with ^{3}H or ^{14}C (min; mean ± SD), was 0.07 ± 0.03 for those made from fish oil (labeled with [^{14}C]cholesterol) and 0.08 ± 0.03 for those made from olive oil (labeled with [^{3}H]cholesterol). The liver radioactive contents, as measured after artificial chylomicron administration, were different among the experimental groups (Table 3). Control rats had the highest liver cholesteryl [1-^{14}C]oleate content, while the fish oil fed group had the lowest. The liver content of [9,10(n)-^{3}H]triolein was lower in fish oil and olive oil fed rats than in control rats. Lower levels of [^{3}H]triacylglycerols were also present in the spleen of control rats as compared to the fish oil and olive oil fed groups. However, it is difficult to interpret the spleen results in view of the extremely low radioactivity values as compared to the liver data. More total fat and triacylglycerol were present in the liver of the olive oil fed group than in the fish oil fed rats; the control livers showed the lowest values (Tables 4 and 5).

DISCUSSION

Our data show that lower liver triacylglycerol output is the major plasma triacylglycerol lowering mechanism of dietary fish oil, which is in agreement with previous reports (7-11, 13-16).

Feeding different fats modified the chylomicron composition, but may also have affected the number of liver chylomicron receptors or lipoprotein lipase activity. These changes may thus have affected the rate of chylomicron metabolism. In order to distinguish between these two alternatives, native chylomicrons containing radioactive cholesterol were obtained from the intestinal lymph of olive oil and of fish oil fed rats and simultaneously infused intra-arterially into recipient rats that had been on a non-purified diet. The metabolism in plasma of chylomicron particles made of olive oil was indistinguishable from that of chylomicron particles made of fish oil, as their FCR were identical. Similar results were suggested in a previous study where infused rat mesenteric lymph chylomicrons had been enriched with either [^{14}C]oleic acid or [^{14}C]eicosapentaenoic acids (18).

Artificial chylomicrons were removed from the plasma at similar rates independent of the diet. Therefore, liver receptors for chylomicrons and the activity of enzymes (lipoprotein lipase) that delipidated the large particles were not influenced by the regular feeding of different types of fats.

The liver is the major organ that metabolizes chylomicron remnants (27,28); therefore after a 10-min period, similar fractions of the injected radioactive artificial chylomicrons should have been found in the liver with all three experimental dietary groups. However, the liver radioactivity values were much higher in the low fat (control) rats than in the other two groups. The liver of fish oil fed rats contained the smallest radioactive concentration of the three groups (Table 3) and much less total fat (Table 4) or triacylglycerol mass than the olive oil

TABLE 3

Liver Radioactivity 10 Minutes After Pulse Arterial Infusion of Artificial Chylomicrons Doubly Labeled with Cholesteryl [1-^{14}C]oleate and [9,10(n) — ^{3}H] Triolein[a]

Experimental groups[b]	Liver		Spleen	
	^{14}CpCE	^{3}H-TG	^{14}C-CE	^{3}H-TG
Controls (n = 10)	22.9 ± 11.4	16.5 ± 8.8	1.1 ± 0.5	0.2 ± 0.1
Fish oil (n = 10)	9.8 ± 1.5	8.0 ± 2.1	1.6 ± 0.8	0.3 ± 0.2
Olive oil (n = 10)	11.7 ± 1.2	9.4 ± 1.3	1.3 ± 0.8	0.4 ± 0.1

[a]Values are expressed as percent of the radioactive dose administered.

[b]Statistical comparisons between groups by Student's t-test were as follows:

Control × fish oil	$p < 0.01$	$p < 0.01$	NS	$p < 0.05$
Control × olive oil	$p < 0.01$	$p < 0.01$	NS	$p < 0.01$
Fish oil × olive oil	$p < 0.01$	NS	NS	NS

TABLE 4

Total Fat in Liver and Spleen (mean ± SD) as Measured at the End of the Artificial Chylomicron Infusion

Experimental groups[a]	Liver		Spleen	
	Total (mg)	mg/g	Total (mg)	mg/g
Controls (n = 10)	277.7 ± 66.1	32.9 ± 7.0	24.0 ± 4.6	35.0 ± 3.2
Fish oil (n = 10)	372.7 ± 54.5	33.8 ± 2.6	14.1 ± 2.9	24.9 ± 3.7
Olive oil (n = 10)	489.3 ± 120.1	43.1 ± 7.8	17.7 ± 4.4	23.5 ± 2.0

[a]Statistical comparisons between groups (mg/g) by Student's t-test were as follows:

Controls × fish oil	NS	$p < 0.001$
Controls × olive oil	NS	$p < 0.001$
Fish oil × olive oil	$p < 0.001$	NS

TABLE 5

Liver Triacylglycerol Mass (mean ± SD) as Measured at the End of the Artificial Chylomicron Infusion

Experimental groups[a]	Total (mg)	mg/g Liver weight
Controls (n = 10)	37.0 ± 8.3	4.3 ± 0.6
Fish oil (n = 10)	45.7 ± 11.5	4.1 ± 0.9
Olive oil (n = 10)	136.7 ± 36.6	12.2 ± 4.1

[a]Statistical comparisons between groups by Student's t-test were as follows:

Controls × fish oil	$p < 0.05$	NS
Controls × olive oil	$p < 0.001$	$p < 0.001$
Fish oil × olive oil	$p < 0.001$	$p < 0.001$

fed rats when values were expressed per unit of liver weight (Table 5).

The present experiments demonstrate that rats fed dietary fish oil rather than olive oil have a slower VLDL-TG production in liver, a normal plasma catabolic rate of radioactive chylomicrons, a decreased liver concentration of the infused radioactive chylomicrons, and a smaller liver concentration of total fat and of triacylglycerol. The data above are in agreement with a slower production of VLDL-TG in the presence of a normal entrance into the liver of the fat dietary origin. If the exit of fat from the liver were impaired while the local synthesis rate of triacylglycerol were maintained, liver fat would have been stored during fish oil feeding. However, the liver fat content was remarkably small in the fish oil fed group compared to that in the olive oil fed group. Therefore, fish oil must stimulate fatty acid oxidation, impair triacylglycerol synthesis, or both.

Previous studies have shown that in the liver ω3 fatty acids are preferentially oxidized as compared to acid of the ω6 series (13,29). Finding little radioactive triacylglycerol in the liver indicates that chylomicrons taken up are more rapidly metabolized into products that are no longer fatty acids; however, slower synthesis of triacylglycerols cannot be ruled out. Dietary fish oil has been found to reduce the activity of certain liver enzymes, such as diacylglycerol hydrolase (30), diacylglycerol acyltransferase (31), phosphatidate phosphohydrolase (29,30), and acetyl-CoA carboxylase (32). Faster oxidation of fish oil derived fatty acids in liver has also been demonstrated in earlier studies (13,33).

Plasma cholesterol concentration was higher in the fish oil group than in the olive oil fed rats (Table 1). Since the fish oil brand used in our study (MaxEPA) contained 4.6 mg cholesterol/g, and the animals consumed approximately 20 g of diet/day, their daily cholesterol intake was approximately 9–10 mg. This intake may have increased the plasma cholesterol concentration in rats (34). In past studies with normal subjects, fish oil treatment did not influence plasma cholesterol concentrations (35,36), but

increased low density lipoprotein (LDL) cholesterol levels (37). Studies with hypercholesterolemic individuals have shown reduced plasma cholesterol concentrations after fish oil treatment (25). The number of LDL receptors was decreased and the number of high density lipoprotein (HDL) receptors was increased in rats fed fish oil as compared to animals fed sunflower oil (38). In rats fed a nonpurified diet, the largest share of plasma cholesterol was found in HDL. Further studies are needed to explain whether and how dietary fish oil influences LDL and HDL metabolism both in humans and in animals.

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Derivatives of Di-*O*-octanoylglycerol and Mono-*O*-octylglycerol as Modulators of Protein Kinase C and Diacylglycerol Kinase Activities

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Twelve analogs of 1,2-di-*O*-octanoylglycerol modified at C-3 and three quaternary *N*-alkyl-ammonium derivatives of glycerol were synthesized. The compounds were tested *in vitro* as potential modulators of the calcium activated, phospholipid dependent protein kinase C (PKC) and diacylglycerol (DAG) kinase activities in order to understand the molecular interactions of these enzymes with their natural activators, inhibitors, or substrates. PKC activity was assayed by measuring histone H_1 phosphorylation, and the compounds synthesized were tested either in the presence (inhibitors) or in the absence (activators) of 1,2-di-*O*-octanoylglycerol analogs with the phosphatidylserine/Ca^{2+} mixture. DAG kinase activity was measured by the incorporation of phosphate into 1,2-di-*O*-oleoyl-*sn*-glycerol in the presence of the various analogs synthesized. In regard to PKC activity, the assays revealed that 1,2-di-*O*-octanoylglycerol analogs are inactive when modified at C-3 with groups which do not permit hydrogen bonding. Under our conditions, di-*O*-octanoylthioglycerol, which has been reported as inactive, was able to activate PKC in the presence of phosphatidylserine. It has been shown to give a synergistic activation with diacylglycerol and had no affinity for the phorbol ester receptor binding site, suggesting that *O*-octanoylthioglycerol interacts with the enzyme at a different site from the phorbol ester receptor binding site. PKC and DAG kinase activities are inhibited by *N*-alkyl-ammonium compounds (IC_{50} 24 μM) only when either two 8-carbon alkyl or acyl chains are present at the 1- and 2-positions of the glycerol backbone. The fact that these compounds have a strong effect on the binding of [^{3}H]phorbol 12,13-dibutyrate to protein kinase C, and also inhibit DAG kinase, may suggest binding to the DAG site of the regulatory domain of PKC.
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Protein kinase C (PKC) is a calcium/phospholipid regulated enzyme which is activated by phorbol esters and diacylglycerols (1). This kinase has emerged as a pivotal regulatory enzyme in cellular signal transduction in response to a large number of external stimuli (2). However, the exact mode of molecular recognition between these activators and the enzyme is still not fully understood (3,4). Previous work has established that 1,2-di-*O*-octanoyl-*sn*-glycerol is an effective activator of PKC activity (5); however, this enzyme also can be activated very efficiently with phorbol ester or with phosphatidylserine (PS) and phosphatidylinositol 4,5-bisphosphate (PIP_2) (6). A number of 1,2-di-*O*-octanoylglycerol analogs have been used to show the importance in the activation process of an hydroxyl group at the 3-position and of the acyl groups at the 1- and 2-positions of the glycerol structure (7,8). On the other hand, natural *O*-alkyl-*O*-acylglycerols and synthetic di-*O*-alkylglycerols also have been shown to inhibit phorbol ester and di-*O*-acylglycerol stimulated PKC activity (9–11). Certain amino compounds may inhibit PKC by interfering with PS, di-*O*-acylglycerol and/or ATP (12–14). After completion of the present work, quaternary ammonium derivatives of *O*-alkylglycerols, with a short chain at the 2-position have been reported to inhibit PKC (15). We therefore reasoned that specific modulators of PKC should be structural analogs of 1,2-*O*-acyl or *O*-alkylglycerol with a function at C-3 that would permit hydrogen bonding as donor or acceptor.

MATERIALS AND METHODS

Materials. Octanoyl chloride and 3-dimethylamino-1,2-propanediol were purchased from Janssen Chimica (Belgium). 3-Amino-1,2-propanediol and 3-mercapto-1,2-propanediol were from Fluka (Mulhouse, France). Other reagents were obtained from Sigma Chimie (L'isle d'Abeau Chesnes, France) or from Aldrich Chimie (Strasbourg, France). The routes of chemical synthesis for compounds shown in Figure 1 are presented in Schemes 1 and 2.

General. In the present paper, "usual work-up" implies dilution of the reaction mixture with dichloromethane and successive washings with ice-cold aqueous solutions of potassium hydrogen sulfate (10%), saturated sodium hydrogen carbonate, and ice-cold water. Aqueous washings were then extracted again with dichloromethane, and the aqueous-washed organic phase was dried (Na_2SO_4) and then evaporated under reduced pressure below 45°C. Thin-layer chromatography (TLC) was done on layers of silica gel (Merck F254, Merck, Darmstadt, Germany) with detection by UV light and/or by charring with H_2SO_4/MeOH/H_2O, and/or dipping into 1% α-cyclodextrin in aqueous methanol (3%), followed by exposure to iodine vapors, and/or dipping into aqueous potassium bichromate (5%) in sulfuric acid (40%). Chromatographic purifications were done under pressure using dry kieselgel 60 (230–400 mesh) columns. Mass spectra were recorded on a Nermag R-10-10C instrument using positive or negative fast-atom bombardment (FAB^+ or FAB^-) with a glycerol matrix, or using DCl/NH_3 techniques. ^{13}C NMR spectra were recorded at 25.18 MHz on a Bruker (Karlsruhe, Germany) WP100 spectrometer. Chemical shifts are given from tetramethylsilane (TMS) in $CDCl_3$ or relative to acetone (31.09 ppm) in D_2O.

3-N-Acetylamino-1,2-di-octanoyloxy-propane (2). 3-Aminopropane-1,2-diol (0.5 g, 5.5 mmol) was selectively *N*-acetylated by treatment at 0°C with acetic anhydride in methanol (7.7 mL, 10% v/v). At 0°C, octanoyl chloride (3.5 mL, 20 mmol) was added dropwise, and methanol

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Abbreviations: ATP, adenosine 5'-triphosphate; DAG, diacylglycerol; 1,2-dioleoyl-*sn*-glycerol; DAG kinase, di-*O*-acylglycerol kinase; DMF, *N,N*-dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FAB, fast-atom bombardment; IC_{50}, concentration causing 50% inhibition; [^{3}H]PDBU, [^{3}H]phorbol 12,13-dibutyrate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PS, phosphatidylserine; TCA, trichloroacetic acid; TLC, thin-layer chromatography; TMS, tetramethylsilane; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

1 X =OH

2 X =$NHCOCH_3$

3 X =$N(CH_3)_2$

4 X =

5 X =

6 X =SH

7 X =$S\text{-}S\text{-}CH_2CH(O_2CC_7H_{15})CH_2O_2C_7H_{15}$

8 X =$SCOCH_3$

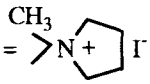
9 X =SCH_3

10 X =F

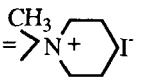
11 CH_2X =$CH(CH_3)OH$

12 X =$N(CH_3)_3{}^+I^-$

13 X =



14 X =



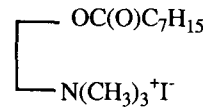


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FIG. 1. Structures of analogs of 1,2-di-*O*-octanoylglycerol or choline.

(5 mL) was added after stirring at room temperature for 12 hr. Evaporation, usual work-up and chromatographic purification (ethyl acetate/hexane, 2:1, v/v) led to **2** in 54% yield. ^{13}C NMR ($CDCl_3$) ppm: 173.5, 173.4 ($CO\text{-}C_7H_{15}$); 170.3 ($CO\text{-}CH_3$); 70.4 ($CH\text{-}O$); 62.8 ($CH_2\text{-}O$); 39.9 ($CH_2\text{-}N$); 34.3, 34.7, 31.7, 29.1, 28.9, 22.6, 14.1 (C_7H_{15}); 23.1 ($CH_3\text{-}CO$). MS (FAB^-) *m/z* 384 $[M - H]^-$; 258 $[M - COC_7H_{15}]^-$. An analytical sample was obtained after bulb-to-bulb distillation (90°C/0.01 mmHg). *Anal.* Calcd for $C_{21}H_{39}NO_5$: C, 65.42; H, 10.19; N, 3.63. Found: C, 65.24; H, 10.26; N, 3.63.

3-N-Dimethylamino-1,2-di-octanoyloxy-propane (**3**). 3-Dimethylaminopropane-1,2-diol (1 g, 8.4 mmol) was esterified as described for the preparation of **2**. Compound **3** was isolated by chromatography (ethyl acetate/hexane, 1:3, v/v) in 91% yield. ^{13}C NMR ($CDCl_3$) ppm: 174.1, 173.4 (CO); 69.4 ($CH\text{-}O$); 64.1 ($CH_2\text{-}O$); 59.6 ($CH_2\text{-}N$); 46.2 (NCH_3); 34.6, 34.3, 31.8, 29.2, 29.1, 25.1, 22.8, 14.2 (C_7H_{15}). MS (FAB^+) *m/z* 372 $[M + H]^+$; 327 $[M + H - N(CH_3)_2]^+$; 244 $[M - COC_7H_{15}]^+$; 226 $[M - HOCOC_7H_{15}]^+$. An analytical sample was obtained after bulb-to-bulb distillation (80°C/0.01 mmHg). *Anal.* Calcd for $C_{21}H_{41}NO_4$: C, 67.88; H, 11.12; N, 3.77. Found: C, 67.78; H, 11.11; N, 3.91.

1,2-Dioctanoyloxy-3-pyrrolidino-propane (**4**). 3-Pyrrolidino-propane-1,2-diol (1 g, 6.8 mmol) was treated as described for the preparation of **2**. Chromatography (ethyl acetate/hexane, 1:4, v/v) afforded **4** in 96% yield. ^{13}C NMR ($CDCl_3$) ppm: 173.6, 173.2 (CO); 70.4 ($CH\text{-}O$); 64.1 ($CH_2\text{-}O$); 56.1 ($CH_2\text{-}N$); 54.7 ($CH_2\text{-}N$); 23.7 ($CH_2\text{-}CH_2$); 34.5, 34.3, 31.8, 29.9, 29.1, 25.1, 22.7, 14.1 (C_7H_{15}). MS (DCl/NH_3) *m/z* 398 $[M + H]^+$. *Anal.* Calcd for $C_{23}H_{43}NO_4$: C, 69.47; H, 10.90; N, 3.52. Found: C, 69.22; H, 10.74; N, 3.50.

1,2-Dioctanoyloxy-3-piperidino-propane (**5**). 3-Piperidino-propane-1,2-diol (1 g, 6.28 mmol) was treated as described for the preparation of **2**. Chromatography (ethyl acetate/hexane, 1:10, v/v) gave **5** (85%). ^{13}C NMR ($CDCl_3$) ppm: 173.5, 173.4 ($CO\text{-}C_7H_{15}$); 69.1 ($CH\text{-}O$); 64.2 ($CH_2\text{-}O$); 58.8 ($CH_2\text{-}N$); 55.0 ($CH_2\text{-}N$); 25.6 ($CH_2\text{-}CH_2N$); 23.8 ($CH_2\text{-}C_2H_4N$); 34.2, 33.9, 31.4, 28.7, 28.6, 24.5, 22.2, 13.5 (C_7H_{15}). MS (DCl/NH_3) *m/z* 412 $[M + H]^+$. *Anal.* Calcd for $C_{24}H_{45}NO_4$: C, 70.03; H, 11.02; N, 3.40. Found: C, 69.76; H, 10.81; N, 3.33.

1,2-Dioctanoyloxy-propane-3-thiol (**6**). This compound was obtained from 1,2-dioctanoyloxy-3,3′-dithio*bis*-propane (**7**). Compound **7** was synthesized as described (16), and obtained in 60% yield after chromatography on silica gel (ethyl acetate/hexane, 1:20, v/v). ^{13}C NMR ($CDCl_3$) ppm: 173.3, 173.1 ($CO\text{-}C_7H_{15}$); 63.6 ($CH_2\text{-}O$); 69.7 ($CH\text{-}O$); 39.5 ($CH_2\text{-}S$); 34.4, 34.2, 31.8, 29.2, 29.1, 25.0, 22.7, 14.2 (C_7H_{15}). MS (FAB^+ PEG200 matrix) *m/z* 718 M^+; 575 $[M\text{-}OCOC_7H_{15}]^+$. *Anal.* Calcd for $C_{38}H_{70}O_8S_2$: C, 63.47; H, 9.81; S, 8.91. Found: C, 63.19; H, 9.94; S, 8.89.

Compound **7** (0.2 g, 0.27 mmol) was dissolved in ethanol (6 mL) and treated with dithioerythrol (86 mg, 0.55 mmol) in the presence of aqueous ammonia (29%, 11 μL). The reaction was carried out at 25°C for 15 min. After evaporation, the residue was dissolved in hexane (20 mL) and washed with water (6 mL, four times). The organic phase was dried and evaporated, and **6** was obtained in 95% yield. ^{13}C NMR ($CDCl_3$) ppm: 173.4, 173.0 ($CO\text{-}C_7H_{15}$); 72.2 ($CH\text{-}O$); 63.1 ($CH_2\text{-}O$); 25.0 ($CH_2\text{-}S$); 34.4, 34.2, 31.8, 29.2, 29.0, 25.0, 22.7, 14.1 (C_7H_{15}). MS (DCl/NH_3) *m/z* 378 $[M + NH_4]^+$; 361 $[M + H]^+$; 217 $[M + H - OCOC_7H_{15}]^+$. An analytical sample was obtained after bulb-to-bulb distillation (85°C/0.01 mmHg). *Anal.* Calcd for $C_{19}H_{36}O_4S$: C, 63.29; H, 10.06; S, 8.89. Found: C, 63.39; H, 10.36; S, 8.97.

3-Acetylthio-1,2-dioctanoyloxy-propane (**8**). Compound **6** (0.1 g, 0.28 mmol) was acetylated with acetic anhydride/pyridine (2 mL, 1:1, v/v). After usual work-up, the residue was purified by chromatography (ethyl acetate/hexane, 1:10, v/v), and **8** was obtained in 80%. ^{13}C NMR ($CDCl_3$) ppm: 173.4, 173.1 ($CO\text{-}C_7H_{15}$); 170.0 ($CO\text{-}CH_3$); 69.9 ($CH\text{-}O$); 63.6 ($CH_2\text{-}O$); 29.6 ($CH_2\text{-}S$); 30.5 ($CH_3\text{-}COS$); 34.3, 34.2, 31.8, 29.2, 29.1, 25.0, 22.7, 14.2 (C_7H_{15}). MS (FAB^+) *m/z* 403 $[M + H]^+$; 327 $[M - SCOCH_3]^+$. *Anal.* Calcd for $C_{21}H_{38}O_5S$: C, 62.65; H, 9.51; S, 7.96. Found: C, 62.89; H, 9.51; S, 8.14.

1,2-Dioctanoyloxy-3-methylthio-propane (**9**). 3-Methylthiopropane-1,2-diol (1 g, 8.18 mmol) was acylated as described for the synthesis of **2**. The expected compound was isolated in 86% yield after chromatography (dichloromethane/hexane, 2:3, v/v). ^{13}C NMR ($CDCl_3$) ppm: 173.4, 173.1 ($CO\text{-}C_7H_{15}$); 70.0 ($CH\text{-}O$); 63.8 ($CH_2\text{-}O$); 34.6

(CH_2-S); 16.2 (CH_3-S) 34.4, 34.2, 31.7, 29.1, 29.0, 25.0, 22.7, 14.2 (C_7H_{15}). MS (DCl/NH_3) *m/z* 392 $[M + NH_4]^+$; 327 $[M - SCH_3]^+$. An analytical sample was obtained after bulb-to-bulb distillation (78-80°C/0.01 mmHg). *Anal.* Calcd for $C_{20}H_{38}SO_4$: C, 64.13; H, 10.22; S, 8.56. Found: C, 64.12; H, 10.23; S, 8.77.

1,2-Dioctanoyloxyl-3-fluoro-propane (**10**). The preparation of 3-fluoro-propane-1,2-diol was carried out following the procedure used by Ghangas and Fondy (17) starting from glycerol. This compound was then acylated as described for 2, and was obtained in 59% yield after chromatography (hexane/diethyl ether, 2:1, v/v). ^{13}C NMR ($CDCl_3$) ppm: 173.6 (*C*O); 81.3 (*C*H_2-F, $J_{CF} = 174$ Hz); 69.6 (*C*H-O, $J_{CF} = 21.7$ Hz); 61.3 (*C*H_2-O, $J_{CF} = 7$ Hz); 34.1, 31.7, 29.1, 28.9, 24.9, 22.6, 14.0 (C_7H_{15}). MS (DCl/NH_3) *m/z* 364 $[M + NH_4]^+$. An analytical sample was obtained after bulb-to-bulb distillation (55°C/0.01 mmHg). *Anal.* Calcd for $C_{19}H_{35}FO_4$: C, 65.86; H, 10.18. Found: C, 65.42; H, 10.13.

3,4-Dioctanoyloxy-butane-2-ol (**11**, *Scheme 1*). Treatment of *meso*-erythrol (7.32 g, 60 mmol) in DMF (50 mL) with 2,2-dimethoxypropane (9.5 mL, 78 mmol) in the presence of *p*-toluenesulfonic acid (165 mg) for 15 min at 80°C afforded after neutralization with triethylamine (4 mL) and chromatography (ethyl acetate/hexane, 1:1, v/v) 3,4-*O*-isopropylidene-butane-1,2-diol (**11a**, 57%). ^{13}C NMR ($CDCl_3$) ppm: 109.4 (*C*$(CH_3)_2$)); 75.8 (*C*H-O); 72.5 (*C*H-OH); 66.5 (*C*H_2-O); 63.7 (*C*H_2-OH); 26.6, 25.2 (C-(*C*$H_3)_2$). MS (DCl/NH_3) *m/z* 163 $[M + H]^+$; 145 $[M - OH]^+$.

Compound **11a** (3.38 g, 20.9 mmol) was dissolved in dichloromethane/pyridine (16 mL; 10:1, v/v) and tosyl chloride (4.3 g, 22 mmol) and pyridine (16 mL) was added dropwise. After stirring for 24 hr at room temperature, the mixture was evaporated to dryness. Usual work-up and chromatography (ethyl acetate/hexane, 1:3, v/v) afforded 3,4-*O*-isopropylidene-1-tosyloxy-butane-2-ol (**11b**) in 78% yield. ^{13}C NMR ($CDCl_3$) ppm: 127.9, 129.9, 132.5 (C_6H_4); 109.4 (*C*-$(CH_3)_2$); 75.1 (*C*H-O); 71.8 (*C*H-OH); 70.6 (*C*H_2-OTS); 66.6 (*C*H_2-O); 26.6, 25.2 (C-(*C*$H_3)_2$); 21.6 (*C*H_3-C_6H_4). MS (DCl/NH_3) *m/z* 334 $[M + NH_4]^+$; 317 $[M + H]^+$; 301 $[M - CH_3]^+$; 131 $[M - CH_2OTS]^+$.

Displacement of the tosyl group of **11b** (4 g, 12.65 mmol) by iodine using sodium iodide (13.7 g, 91 mmol) in DMF (50 mL) was achieved by stirring for 4 hr at 80°C. Usual work-up and chromatography (ethyl acetate/hexane, 1:3, v/v) afforded 1-iodo-3,4-*O*-isopropylidene-butane-2-ol **11c** in 64% yield. ^{13}C NMR ($CDCl_3$) ppm: 109.3 (*C*-*C*$H_3)_2$); 77.7 (*C*H-O); 72.1 (*C*H-OH); 66.7 (*C*H_2-O); 26.9, 25.2 (C-(*C*$H_3)_2$); 12.7 (*C*H_2-I).

Compound **11c** (0.5 g, 1.9 mmol) was dissolved in ethanol (20 mL) containing triethylamine (0.8 mL) and was hydrogenated in the presence of Pd/C (10%, 0.5 g, 6 at.) for 20 hr at room temperature. After removing the catalyst, evaporation of the solvent and purification by gel chromatography (ethyl acetate/hexane, 1:3, v/v), 3,4-*O*-isopropylidene-butane-2-ol (**11d**) was isolated in 61% yield. ^{13}C NMR ($CDCl_3$) ppm: 109.3 (*C*-$(CH_3)_2$); 79.6 (*C*H-O); 67.1 (*C*H_2-OH); 64.8 (*C*H_2-O); 26.6, 25.4 (C-(*C*$H_3)_2$); 18.5 (*C*H_3). MS (DCl/NH_3) *m/z* 164 $[M + NH_4]^+$; 147 $[M + H]^+$; 131 $[M - CH_3]^+$.

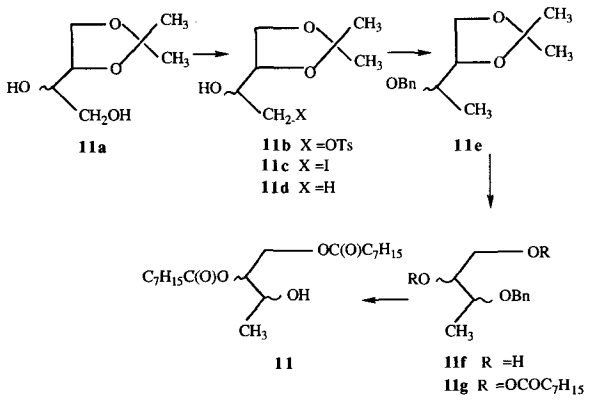
Compound **11d** (0.1 g, 0.68 mmol) was dissolved in DMF (7 mL). Sodium hydride (0.75 mmol) was added followed by benzyl bromide (97 μL, 0.8 mmol) and tetrabutylammonium iodide (25 mg) as described (18). The mixture was stirred for 12 hr at room temperature. Evaporation, usual work-up, and chromatography (ethyl acetate/hexane, 0.5:20, v/v) led to 3-benzyloxy-1,2-*O*-isopropylidene-butane (**11e**, 75% yield). ^{13}C NMR ($CDCl_3$) ppm: 139.4, 129.1, 128.4 (C_6H_5); 109.7 (*C*-$(CH_3)_2$); 79.2 (*C*H-O*C*H_2); 75.8 (*C*H-O); 71.2 (O-*C*H_2-C_6H_5); 66.9 (*C*H_2-O); 26.2, 24.9 (C-(*C*$H_3)_2$; 15.8 (*C*H_3). MS (El) *m/z* 236 M^+; 221 $[M - CH_3]^+$; 178 $[M - OC(CH_3)_2]^+$; 91 $[CH_2C_6H_5]^+$.

Tetrafluoroboric acid (35%) in H_2O (119 μL, 0.59 mmol) was added to a stirred solution of compound **11e** (93 mg, 0.39 mmol) in acetonitrile (5.5 mL) as described (19). After stirring for 30 min at room temperature, triethylamine (120 μL) was added, and the solvent was evaporated. The residue gave after chromatography (ethyl acetate/hexane, 1:1, v/v) 3-benzyloxy-butane-1,2-diol (**11f**) in 87% yield. ^{13}C NMR (D_2O) ppm: 138.4, 129.4, 128.9 (C_6H_5); 76.3 (*C*H-O*C*H_2); 74.5 (*C*H-OH); 71.5 (O-*C*$H_2C_6H_5$); 63.3 (*C*H_2-OH); 15.2 (*C*H_3). MS (DCl/NH_3) *m/z* 214 $[M + NH_4]^+$; 197 $[M + H]^+$; 91 $[CH_2C_6H_5]^+$.

3-Benzyloxy-1,2-di-octanoyloxy-butane (**11g**). **11f** afforded after treatment with octanoyl chloride, as described for **2**, and chromatography (ethyl acetate/hexane, 0.5:20, v/v) compound **11g** in 53% yield. ^{13}C NMR ($CDCl_3$) ppm: 163.7, 163.2 (*C*O); 138.0, 128.6, 127.9 (C_6H_5); 73.7, 73.6 (*C*H-O); 71.4 (O-*C*H_2-O); 34.6, 34.3, 31.9, 29.3, 29.1, 25.1, 22.7, 14.2 (C_7H_{15}). MS (DCl/NH_3) *m/z* 466 $[M + NH_4]^+$; 449 $[M + H]^+$; 305 $[M - OCOC_7H_{15}]^+$.

3,4-Dioctanoyloxy-butane-2-ol (**11**). Catalytic hydrogenation (6 at.) over Pd/C (10%, 94 mg) for 24 hr in ethyl acetate (20 mL) of **11g** (94 mg, 0.2 mmol) led to **11** (52%) contaminated with its 2,4 isomer (20%, as estimated by NMR). ^{13}C NMR ($CDCl_3$) ppm: 174.1, 173.4 (*C*O); 75.4 (*C*H-O); 66.9 (*C*H-OH); 62.3 (*C*H_2-O) 19.0 (*C*H_3); 34.6, 34.3, 31.8, 29.2, 25.1, 22.8, 14.2 (C_7H_{15}). MS (DCl/NH_3) *m/z* 376 $[M + NH_4]^+$; 359 $[M + H]^+$; 341 $[M + H - H_2O]^+$. *Anal.* Calcd for $C_{20}H_{38}O_5$: C, 67.00; H, 10.68. Found: C, 67.08; H, 10.59.

*N-(1,2-Dioctanoyloxy-3-propyl-*N,N,N-*trimethylammonium iodide* (**12**). To a cold solution of compound **3** (0.3 g, 0.8 mmol) in diethyl ether (10 mL) was added methyl iodide (0.25 mL, 4 mmol). After a few minutes at room temperature, the compound which separated was filtered off by suction and recrystallized from ether at 4°C (54%; m.p. 142-144°C). ^{13}C NMR ($CDCl_3$) ppm: 173.2, 172.8



SCHEME 1

(*CO*); 66.2 (*CH*$_2$-N) 65.9 (*CH*-O); 63.0 (*CH*$_2$-O) 54.9 (N(*CH*$_3$)$_3$); 34.3, 34.1, 31.7, 29.1, 29.0, 24.7, 22.6, 14.1 (*C*$_7$H$_{15}$). MS (FAB$^-$) *m/z* 512 [M − H]$^-$. *Anal.* Calcd for $C_{22}H_{44}INO_4$: C, 51.46; H, 8.63; I, 24.71; N, 2.72. Found: C, 51.73; H, 8.63; I, 24.94; N, 2.75.

N-(1,2-Dioctanoyloxy-3-propyl)-N-methyl-N,N-pyrrolidinium iodide (**13**). Alkylation of compound **4** was performed in a manner analogous to that of **3**. The expected compound **13** was obtained in 42% yield after crystallization from diethyl ether/hexane; m.p. 62-64°C. ^{13}C NMR ($CDCl_3$) ppm: 173.2, 172.8 (*CO*); 66.3 (*CH*$_2$-N*CH*$_2$); 65.8 (*CH*-O); 63.6 (*CH*$_2$-O); 63.0 (*CH*$_2$-N); 49.5 (*CH*$_3$-N); 21.9, 21.2 (*CH*$_2$-*CH*$_2$N); 34.2, 34.0, 31.6, 29.0, 28.9, 24.7, 24.6, 22.6, 14.0 (*C*$_7$H$_{15}$). MS (DCl/NH$_3$) *m/z* 540 [M + H]$^+$; 398 [M + H − I − CH$_3$]$^+$. *Anal.* Calcd for $C_{24}H_{46}INO_4$: C, 53.42; H, 8.59; I, 23.52; N, 2.59. Found: C, 53.51; H, 8.67; I, 23.31; N, 2.58.

N-(1,2-Dioctanoyloxy-3-propyl)-N-methyl-N,N-piperidinium iodide (**14**). Alkylation of compound **5** as described for compound **3** afforded **14** (30%; m.p. 70-73°C). ^{13}C NMR ($CDCl_3$) ppm: 173.2, 172.8 (*CO*); 65.3 (*CH*-O); 63.4 (*CH*$_2$-O); 62.4, 62.3 (*CH*$_2$-N); 49.2 (*CH*$_3$-N); 24.9, 20.4 (*CH*$_2$-*CH*$_2$N); 34.3, 34.1, 31.7, 29.1, 22.7, 14.1 (*C*$_7$H$_{15}$). MS (DCl/NH$_3$) *m/z* 554 [M + H]$^+$; 412 [M + H − I − CH$_3$]$^+$. *Anal.* Calcd for $C_{25}H_{48}INO_4$; C, 54.24; H, 8.74; I, 22.92; N, 2.53. Found: C, 55.24; H, 8.74; I, 22.42; N, 2.49.

N-(1-Octanoyloxy-2-ethyl)-N,N,N-trimethylammonium iodide (**15**). To a solution of 2-*N,N*-dimethylamino-ethanol (5 g, 56 mmol) in diethyl ether (20 mL) was added octanoyl chloride (9.59 mL, 56 mmol) and the reaction was stirred overnight at room temperature. The precipitate was collected and dried to give 2-*N,N*-dimethylamino-1-octanoyl-ethane hydrochloride (98%). ^{13}C NMR ($CDCl_3$) ppm: 173.1 (*CO*); 58.2 (*CH*$_2$-O); 56.1 (*CH*$_2$-N); 43.5 (N-(*CH*$_3$)$_2$); 34.0, 31.6, 29.0, 28.9, 24.7, 22.6, 14.0 (*C*$_7$H$_{15}$). *Anal.* Calcd for $C_{12}H_{26}CINO_2 \times 0.5H_2O$: C, 55.26; H, 10.43; N, 5.37. Found: C, 55.49; H, 10.17; N, 5.40. The salt was stirred in methanol with Dowex (OH)$^+$ resin for a few minutes. After filtration and evaporation, the compound was treated as already described for the synthesis of **12**. Compound **15** was obtained in 47% yield (m.p. 132-134°C). *Anal.* Calcd for $C_{13}H_{28}INO_2 \times 0.5H_2O$: C, 42.63; H, 7.98; I, 34.64; N, 3.82. Found: C, 42.72; H, 7.86; I, 34.40; N, 3.91.

2,3-O-Isopropylidene-1-octyloxy-propane-2,3-diol (**17**). This compound was obtained following the procedure by Baumann and Mangold (20), but starting with octyl methanesulfonate and glycerol derivative **16** and using toluene as solvent. The expected compound **17** was obtained in 76% yield after chromatography (hexane/ethyl acetate, 40:1, v/v). ^{13}C NMR ($CDCl_3$) ppm: 109.8 (*C*(CH$_3$)$_2$); 74.9 (*CH*-O); 71.9 (*CH*$_2$-O-*CH*$_2$); 67.0 (*CH*$_2$-O); 25.6, 25.0 (C(*CH*$_3$)$_2$); 31.5, 29.2, 29.0, 28.8, 26.3, 22.2, 13.5 (*C*$_7$H$_{15}$). MS (EI) *m/z* 245 [M + H]$^+$; 229 [M − CH$_3$]$^+$.

3-Octyloxy-propane-1,2-diol (**18**). Compound **17** (0.6 g, 2.5 mmol) was treated at 100°C in acetone (2 mL) in the presence of aqueous 1N HCl (3 mL) for 30 min. After neutralization, 1-octylglycerol **18** can be used without purification for the next step. An analytical sample is obtained by filtration through silica gel (hexane/ethyl acetate, 3:1, v/v). ^{13}C NMR ($CDCl_3$) ppm: 72.5 (*CH*$_2$-OC$_8$H$_{17}$); 72.0 (O-*CH*$_2$-C$_7$H$_{15}$); 70.8 (*CH*-OH); 64.3 (*CH*$_2$-OH); 32.0, 29.7, 29.6, 29.4, 26.2, 22.8, 14.2 (*C*$_7$H$_{15}$). MS (DCl/NH$_3$) *m/z* 222 [M + NH$_4$]$^+$; 205 [M + H]$^+$. *Anal.* Calcd for $C_{11}H_{24}O_3$; C, 64.66; H, 11.84. Found: C, 64.45; H, 12.10.

1-Octyloxy-3-tosyloxy-propane-2-ol (**19**). The above compound **18** (2.4 mmol) was dissolved in pyridine (5 mL), chilled at 4°C, and tosyl chloride (1.5 eq.) was added. The resulting mixture was stirred for 20 hr at room temperature. After usual work-up, **19** was obtained in 37% yield after chromatography (hexane/ethyl acetate, 9:1, vwv). ^{13}C NMR ($CDCl_3$) ppm: 145.9, 130.6, 128.7 (*C*$_6$H$_4$); 71.9, 70.8 (*CH*$_2$-O-*CH*$_2$); 70.6 (*CH*-OH); 68.4 (*CH*$_2$-OTS); 21.1 (*CH*$_3$-C$_6$H$_4$); 31.4, 29.1, 28.8, 25.6, 22.2, 13.5 (*C*$_7$H$_{15}$). MS (DCl/NH$_3$) *m/z* 376 [M + NH$_4$]$^+$; 359 [M + H]$^+$; 187 [M − OTS]$^+$. *Anal.* Calcd for $C_{18}H_{30}O_5S$: C, 60.30; H, 8.43; S, 8.94. Found: C, 60.17; H, 8.36; S, 8.71.

1-Azido-3-octyloxy-propane-3-ol (**20**). The displacement of the tosyl group was achieved by treatment of **19** (0.25 g, 0.69 mmol) with lithium azide (0.13 g, 2.8 mmol) in DMF (10 mL) at 60°C for 12 hr. The solvent was evaporated; usual work-up of the residue and chromatography (ethyl acetate/hexane, 1:9, v/v) afforded **20** (95%). ^{13}C NMR ($CDCl_3$) ppm; 72.0 (*CH*$_2$-O-*CH*$_2$); 69.8 (CH-OH); 53.7 (*CH*$_2$-N$_3$); 32.0, 29.7, 29.6, 29.4, 26.2, 22.8, 14.2 (*C*$_7$H$_{15}$). MS (DCl/NH$_3$) *m/z* 247 [M + NH$_4$]$^+$; 230 [M + H]$^+$.

1-Azido-2-methyloxy-3-octyloxy-propane (**21**). Compound (**20**) (60 mg, 0.26 mmol) was treated with sodium hydride (12 mg, 50% in oil dispersion) in DMF (3 mL). After 30 min at room temperature, methyl iodide (0.28 mmol) was added and the mixture was heated at 50°C for 12 hr, diluted with dichloromethane, and washed with ice-cold water. Compound **21** was obtained in 68% yield after chromatography (hexane/ethyl acetate, 15:1, v/v). ^{13}C NMR ($CDCl_3$) ppm: 79.6 (*CH*-OCH$_3$); 72.0, 69.9 (*CH*$_2$-O-*CH*$_2$); 51.9 (*CH*$_2$-N$_3$); 32.0, 29.8, 29.6, 29.4, 26.2, 22.9, 14.2 (*C*$_7$H$_{15}$). MS (DCl/NH$_3$) *m/z* 261 [M + NH$_4$]$^+$; 244 [M + H]$^+$.

1-N,N-Dimethylamino-2-methoxy-3-octyloxy-propane (**22**). The azido compound **21** (66 mg, 0.27 mmol) was treated as described (21) for 12 hr with pyridine/ammonia-saturated methanol (3.6 mL; 1:1, v/v) in the presence of triphenyl phosphine (180 mg). The reaction mixture was diluted with diethyl ether (50 mL) and washed with aqueous 1N HCl (50 mL). To the aqueous phase, NaOH (1N) was added (until slightly basic), and this phase was extracted with chloroform (3 times). The organic layer was dried, filtered and concentrated to yield the expected amino compound in 73% yield. This compound was used for the next step without characterization and was *N,N*-dimethylated following the procedure recently described (22). Compound **22** was obtained in 70% after chromatography (ethyl acetate/methanol, 3:7, v/v). ^{13}C NMR ($CDCl_3$) ppm: 78.9 (*CH*-OCH$_3$), 71.9 (*CH*$_2$-O-*CH*$_2$-C$_7$H$_{15}$); 61.1 (*CH*$_2$-N); 57.9 (O-*CH*$_3$); 46.3 (N(*CH*$_3$)$_2$); 32.0, 29.9, 29.6, 29.4, 26.3, 22.8, 14.1 (*C*$_7$H$_{15}$). MS [FAB$^+$] *m/z* 246 [M + H]$^+$.

N-(1-Octyloxy-2-methoxy-3-propyl)-N,N,N-trimethylammonium iodide (**23**). This compound was prepared in 80% yield as described for the synthesis of **12** (m.p. 99°C). ^{13}C NMR ($CDCl_3$) ppm: 74.7 (*CH*-O); 72.1 (O-*CH*$_2$-C$_7$H$_{15}$); 68.1 (CH$_2$-O); 67.6 (CH$_2$-N); 58.5 (O-CH$_3$); 55.4 (N(*CH*$_3$)$_3$); 31.8, 29.4, 29.2, 26.0, 22.6, 14.0 (*C*$_7$H$_{15}$). MS (FAB$^-$) *m/z* 514 [M + I]$^-$; 127 [I]$^-$; (FAB$^+$) *m/z* 260 [M − I]$^+$. *Anal.* Calcd for $C_{15}H_{34}INO_2$; C, 46.51; H, 8.84; I, 32.76; N, 3.61. Found: C, 46.62; H, 8.93; I, 32.20; N, 3.52.

1-Amino-3-octyloxy-propane-2-ol (**24**). Reduction of the azido group of **20** (75 mg, 0.32 mmol) was done as described for compound **21**. Compound **24** was obtained in

93% yield. ^{13}C NMR ($CDCl_3$) ppm: 77.1 (*C*H-OH); 73.2 (*C*H_2-O); 71.9 (*C*H_2-C_7H_{15}); 44.6 (*C*H_2-N); 31.9, 29.8, 29.5, 26.3, 22.8, 14.2 (C_7H_{15}). MS (DCI/NH_3) *m/z* 204 $[M + H]^+$; 185 $[M - NH_3]^+$.

*2-Acetyloxy-1-*N,N-*dimethylamino-3-octyloxy-propane* (25). Compound 24 was *N,N*-dimethylated as already described for the synthesis of 22, the resulting product was acetylated with acetic anhydride/pyridine mixture (1:1, v/v), and 25 was obtained in 65% yield after purification (ethyl acetate/methanol, 7:3, v/v). ^{13}C NMR ($CDCl_3$) ppm: 171.0 (*C*O); 71.6 (*C*H-O); 70.5, 70.6 (*C*H_2-O-*C*H_2); 59.7 (*C*H_2-N); 46.0 (N(*C*H_3)$_2$) 21.3 (*C*H_3); 31.8, 29.6, 29.4, 29.2, 26.0, 22.6, 14.0 (C_7H_{15}). MS (FAB^+) *m/z* 274 $[M + H]^+$.

N-*(2-Acetyloxy-1-octyloxy-3-propyl)*-N,N,N-*trimethylammonium iodide* (26). *N*-Alkylation of compound 24 was accomplished as described for the synthesis of 23. Compound 26 was obtained in 82% yield (m.p. 128–129°C). ^{13}C NMR ($CDCl_3$) ppm: 170.3 (*C*O); 72.3 (*C*H-O); 67.6 (*C*H_2-O-*C*H_2); 67.1 (*C*H_2-N); 55.1 (N(*C*H_3)$_3$); 21.4 (*C*H_3); 32.0, 29.7, 29.5, 29.4, 26.2, 22.8, 14.2 (C_7H_{15}). MS (FAB^-) *m/z* 542 $[M + I]^-$; (FAB^+) *m/z* 288 $[M - I]^+$. *Anal.* Calcd for $C_{16}H_{34}INO_3$: C, 46.26; H, 8.25; I, 30.55; N, 3.37. Found: C, 46.21; H, 8.38; I, 30.30; N, 3.30.

*1-*N,N-*Dimethylamino-2-octanoyloxy-3-octyloxy-propane* (27). Compound 24 (63 mg, 0.3 mmol) was *N,N*-dimethylated as already described for the synthesis of 22, and the resulting product was acylated with octanoyl chloride (1.3 eq; 62 μL) in pyridine/dichloromethane (3:1, v/v). Usual work-up and purification (hexane/ethyl acetate, 7:3, v/v) afforded 27. ^{13}C NMR ($CDCl_3$) ppm: 173.4 (*C*O); 71.5 (*C*H-O); 70.6, 70.4 (*C*H_2-O-*C*H_2); 46.0 (N(*C*H_3)$_2$); 34.5, 31.8, 31.7, 29.6, 29.4, 29.2, 29.1, 29.0, 26.0, 25.0, 22.6, 22.5, 14.1, 14.0 (C_7H_{15}). MS (EI) *m/z* 358 $[M + H]^+$; 213 $[M - OCOC_7H_{15}]^+$. *Anal.* Calcd for $C_{21}H_{43}NO_3$: C, 70.53; H, 12.12; N, 3.91. Found: C, 70.48; H, 12.38; N, 4.0.

N-*(2-Octanoyloxy-1-octyloxy-3-propyl)*-N,N,N-*trimethylammonium iodide* (28). *N*-Alkylation of compound 27 was performed as described for the synthesis of 23. Compound 28 was obtained in 38% yield after crystallization in diethyl ether (m.p. 126–128°C). ^{13}C NMR ($CDCl_3$) ppm: 172.9 (*C*O); 72.0 (*C*H_2-C_7H_{15}); 69.4 (*C*H-O); 67.3 (*C*H_2-O); 66.6 (*C*H_2-N); 54.7 (N(*C*H_3)$_3$); 34.3, 31.8, 31.6, 29.4, 29.3, 29.2, 29.0, 28.8, 26.0, 24.7, 22.6, 22.5, 14.1, 14.0 (C_7H_{15}). MS (FAB^-) *m/z* 626 $[M + I]^-$; (FAB^+) *m/z* 373 $[M - I]^+$. *Anal.* Calcd for $C_{22}H_{46}INO_3$: C, 52.89; H, 9.28; I, 25.4; N, 2.80. Found: C, 53.10; H, 9.47; I, 25.50; N, 2.90.

Protein kinase C. Protein kinase C was purified from bovine brain according to Walton *et al.* (23). A final specific activity of 90 nmol of phosphorus transferred per min per mg protein was obtained. Protein kinase C was routinely assayed by measuring ^{32}P transferred from [γ^{32}P]ATP to histone H_1 according to Vilgrain *et al.* (24). Briefly, the reaction was run in a final volume of 80 μL of 50 mM Tris-HCl, pH 7.4, containing 50 ng of enzyme, $CaCl_2$ 100 μM, $MgCl_2$ 10 mM, histone H_1 37.5 μg/mL, phosphatidylserine 31 μM, and 1,2-dioleoyl-*sn*-glycerol 0.5 μM. Phosphatidylserine and 1,2-dioleoyl-*sn*-glycerol were dispersed by sonication in 50 mM Tris-HCl, pH 7.4, containing 0.5 mM ethylenediaminetetraacetic acid (EDTA). Synthetic compounds were dissolved in dimethylsulfoxide (final concentration, 0.1%). After addition of 10 μM [γ^{32}P]ATP (1250 cpm/pmol), the sample was further incubated for 10 min at 30°C. The reaction was terminated by the addition of 12% trichloroacetic acid (TCA) in the presence of casein as carrier protein. After centrifugation for 5 min at 5000 rpm, the supernatant was discarded and the pellet was washed twice with 2.5 mL of TCA. The final pellet was resuspended in 100 μL of 1N NaOH, and ^{32}P incorporation was measured by scintillation counting in Aquasol (New England Nuclear, Boston, MA).

[^{3}H]PDBU binding assay. [^{3}H]PDBU binding was measured using the polyethylene glycol precipitation method (25). The reaction was carried out in a final volume of 100 μL of 50 mM Tris-HCl, pH 7.4, containing 0.5 mg of purified PKC, $CaCl_2$ 100 μM, bovine serum albumin 0.2 mg/mL, phosphatidylserine 250 μg/mL, and [^{3}H]-PDBU 10 nM (15.8 Ci/mmol) for 30 min at 30°C and then cooled to 4°C for 1 hr to ensure precipitation by polyethylene glycol 6000 (final concentration, 12%) in the presence of bovine γ-globuline (1 mg). After centrifugation at 14,000 rpm, the pellet was dissolved in 20 μL of 1N NaOH and the radioactive [^{3}H]PDBU was determined by scintillation counting. Non-specific binding was measured in the presence of 30 μM unlabeled PDBU.

Diacylglycerol kinase assay. DAG kinase was purified from pig brain according to Kanoh *et al.* (26). A final specific activity of 0.8 μmol of phosphatidate produced per min per mg protein was obtained. The diacylglycerol kinase assay was carried out in a final volume of 40 μL 100 mM Tris-HCl, pH 7.5, buffer containing 10 mM NaF, 1 mM dithiothreitol (DTT), 8 mM $MgCl_2$, 0.5 mM deoxycholate, 5 μM diolein, 200 μM [γ^{32}P]ATP (125 cpm/pmol) and 50 ng of enzyme. Synthetic compounds were dissolved in DMSO (final concentration, <0.1%). Labelled phosphatidate was extracted as described (27) and counted by scintillation counting.

RESULTS AND DISCUSSION

The aim of this study was to investigate both the ability of analogs of di-*O*-octanoylglycerol (1) to act as specific activators, inhibitors or substrates of PKC and DG kinase, and to examine the possible involvement of the 3-hydroxyl group of 1,2-diacylglycerols in bringing about these efforts. All compounds studied were racemic mixtures. Hence, no information was acquired as to the relative activities of the enantiomers.

Ganong *et al.* (8) suggested the involvement of the 3-hydroxyl group by demonstrating that a deoxy compound, or substitution of this group by chloride or by bulky groups produced inactive compounds. Earlier efforts (28, 29) to rationalize structure-activity relationships of potent activators of PKC had been based on their conformational properties. Also, Brockerhoff (3) had suggested formation of an intermediary complex involving the 3-hydroxy group as a proton donor to the enzyme. It also was proposed that the ester groups acted as acceptors of protons from the protein. Since a hydroxyl group can act as a proton acceptor and can donate its hydrogen to a proton acceptor, such as the solvent or a protein, we considered it of interest to examine the effect of substitution of the hydroxyl group with groups that are polar, apolar or amphiphilic in characters.

Substituted nitrogen derivatives were prepared first. These compounds (2–5) were easily obtained by acylation of commercially available precursors. All were inactive under our experimental conditions (IC 50 > 100 μM). The

sulfur containing compounds 6–9 were readily synthesized. Although compounds 7–9 were inactive, compound 6 with a free thiol at C-3 proved to be a potent activator of PKC (Fig. 2A). At a concentration of 40 μM, both diacylglycerol and thiol 6 produced about the same activation with A_{50} values of approximately 5 μM and 18 μM, respectively. For compound 6, the dose-dependent activation was without saturation up to 40 μM.

Compound 6 had been tested previously as activator or antagonist of PKC. However, it was reported to be inactive (8). A likely explanation for this would be that the thiol had undergone autoxidation to disulfide 7, which we have found inactive. Although compound 6 proved to be first analog of di-*O*-octanoylglycerol 1 which significantly promoted PKC activation, the compound did not displace the [^{3}H]PDBU binding at concentrations up to 30 μM (Fig. 2B). This conclusion was supported by the synergistic action of the thiol with diacylglycerol. Activation of PKC was observed (Fig. 2C, curve a) when compound 6 was used at constant concentration (20 μM) and diacylglycerol was added in a concentration range of 0–40 μM. The theoretical curve for an additive effect is represented by curve b. The lack of coincidence of these plots suggests that 6 binds to a site different from the PDBU binding site.

Racemic mixtures of the fluoro compound 10 and the C-methyl derivative of 1 (11) were essentially inactive. During our work, the optically pure isomers of 11 were measured by Wender *et al.* (30) and only one isomeric form was shown to be slightly active. In view of the inactivity displayed by fluoride 10, the hydroxyl group of 1 appears to donate its hydrogen to hydrogen bonding with the receptor site responsible for PKC activation.

Structurally dissimilar hydrophobic compounds with free or protonated amino groups have been reported to be potent inhibitors of PKC by interfering with phosphatidylserine (12,31). Furthermore, very recently quaternary ammonium analogs of alkyl glycerols with short alkyl chains at C-2 were found to reverse PS activation of PKC (15). These compounds are structurally related to choline phosphoglycerides and their *in vivo* turnover does not involve the phosphatidylinositol cycle. Keeping in mind a selective regulation of this pathway, we have synthesized a series of quaternary ammonium derivatives of 1,2-di-*O*-octanoylglycerols 12–14 (Fig. 1), a 1,2-di-*O*-alkyl-glycerol (23) and 1-*O*-alkyl-2-*O*-acyl-glycerols (26 and 28) (Scheme 2). The choline analog 15 also was prepared. All these cationic compounds were tested for *in vitro* inhibition of PKC and DG kinase. The results, shown in Table 1, indicate that the activation of all these compounds with two C-8 chains were not significantly different, in that all caused a half-maximum inhibition at ~20 μM for both enzymes. As expected, substitution of an acyl chain of 12 by an alkyl chain at C-1 to produce 28 did not alter the activity. However, contrary to the reported behavior of *O*-alkylglycerol derivatives (15), both the 2-*O*-methyl and 2-*O*-acetyl analogs of 28 (23 and 26) were inactive under our conditions, as was compound 15. As shown in Figure 3, the inhibition of PKC is due to competition for the receptor site for 1. Since [^{3}H]PDBU binding was inhibited by 12–14 and 28 in a dose-dependent fashion, it seems unlikely that the inhibitory effects described in this paper can be explained by either detergent effects or by competition with other agents (ATP, PS, or substrates). The results appear to be

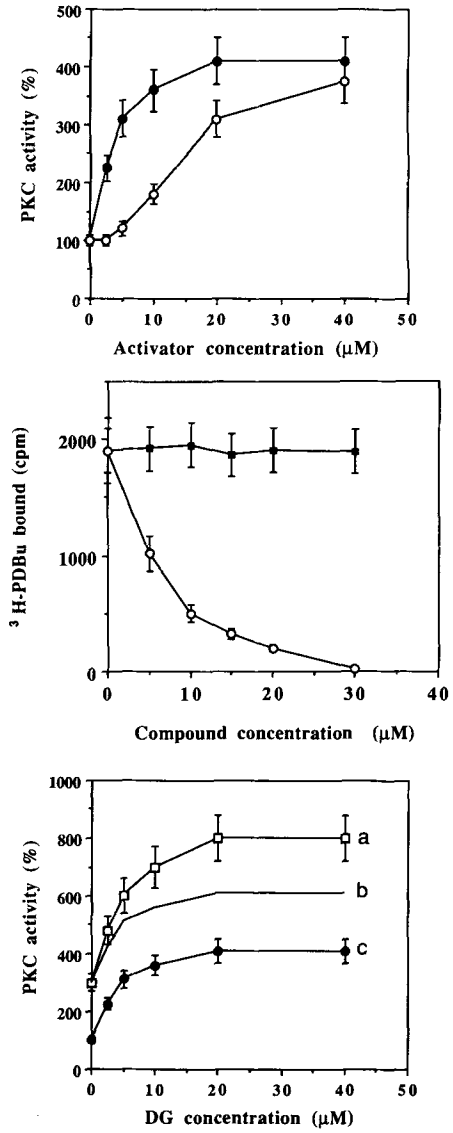
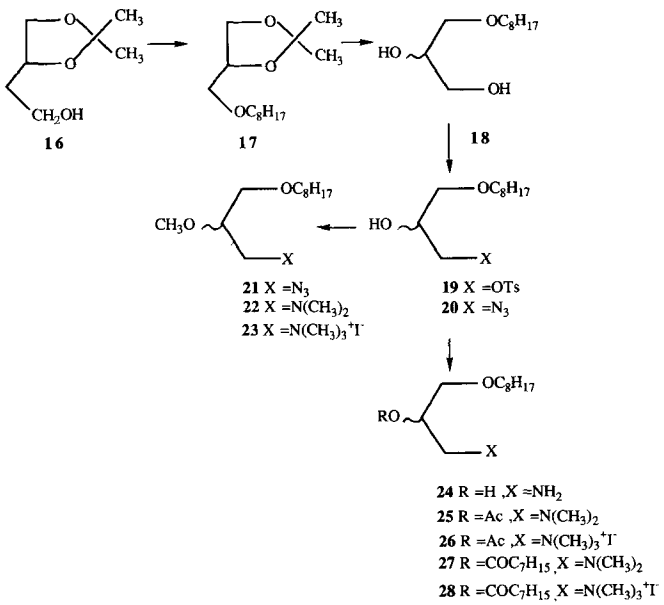


FIG. 2. Effect of 1,2-di-*O*-acylglycerol and 1,2-di-*O*-octanoyl-thioglycerol on PKC activity and [^{3}H]PDBU binding. A. PKC activity was assayed as described in Materials and Methods. Enzyme (500 ng) was incubated in the presence of [γ^{32}P]ATP and histone H_1 for 20 min at 30°C with increasing concentrations of 1,2-di-*O*-acylglycerol (closed circle) or 1,2-di-*O*-octanoyl-thioglycerol 6 (open circle) from 0–40 μM. Incorporation of radioactivity into H_1 was counted after TCA precipitation with casein as carrier protein. The results are expressed as % of control where control is the enzyme activity in the presence of phosphatidylserine/Ca^{2+}. B. [^{3}H]PDBU binding was assayed as described in Materials and Methods in a final volume of 100 μL containing 500 μg of enzyme, phosphatidylserine 250 μg/mL, 10 nM [^{3}H]PDBU (15.8 Ci/mol), and 100 μM $CaCl_2$ in the presence of increasing concentrations of di-*O*-acylglycerol (open circle) or 1,2-di-octanoyl-thioglycerol 6 (closed circle) from 0–30 μM. Specific binding was determined by counting [^{3}H]PDBU after PEG 6000 precipitation in the presence of bovine-γ globuline as carrier protein. Results are expressed as cpm of [^{3}H]PDBU counted. Data points are the mean of triplicate determinations ± SEM as indicated. C. Effects of 1,2-di-*O*-octanoyl-thioglycerol on PKC activation by 1,2-di-*O*-acylglycerol. The enzyme activity was determined as described in A with increasing concentrations of 1,2-di-*O*-acylglycerol, in the absence of compound 6 (closed circle) (curve c), or in the presence of 20 μM thioglycerol (open square) (curve a); curve b is the theoretical curve for an additive effect. Data are expressed as % of control where control is the PKC activity in the presence of phosphatidylserine/Ca^{2+}.



SCHEME 2

TABLE 1

Effect of *N*-Alkylammonium Derivatives of 1,2-Di-*O*-octanoylglycerol on PKC and DAG Kinase Activities[a]

Compounds	IC_{50} (μM) PKC	DAG K
12	23	18
13	25	30
14	20	20
15	>100	>100
23	>100	>100
26	>100	>100
28	20	20

[a]PKC activity was tested as described in Materials and Methods using a mixture of *sn*-1,2-diolein/PS/Ca^{2+} activators + the indicated compounds. DAG kinase activity was assayed using *sn*-1,2-diolein as substrate in the presence of the compounds indicated.

best explained by interactions at or near the DAG binding site.

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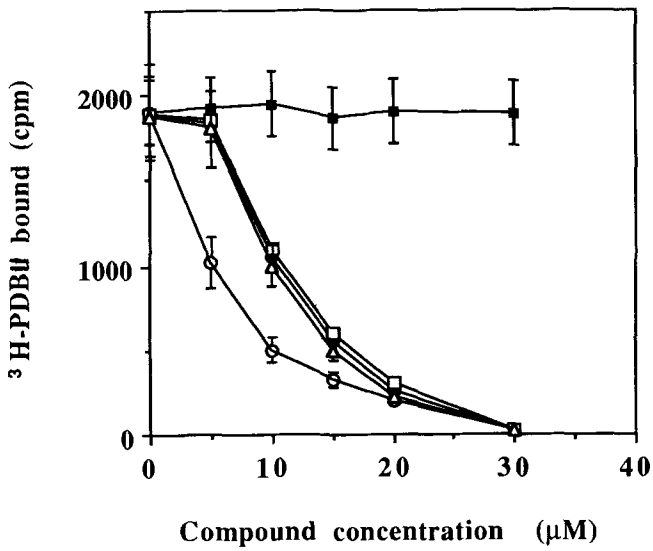

FIG. 3. Effect of quaternary ammonium compounds on [^{3}H]PDBU binding of PKC. [^{3}H]PDBU binding was assayed as described in Materials and Methods by incubating 500 μg of enzyme with phosphatidylserine/Ca^{2+} and in the presence of increasing concentrations of 15, 23, 26 (closed square); 13 (open triangle); 12, 28 (open square); 14 (closed cirlce); 1,2-di-*O*-acylglycerol (open circle). Specific bonding was counted after PEG 6000 precipitation in the presence of bovine-γ globuline as carrier protein. Results are expressed as cpm of [^{3}H]PDBU counted. Data are mean values of 3 determination ± SEM as indicated.

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Effect of Dietary Fats on Some Membrane-Bound Enzyme Activities, Membrane Lipid Composition and Fatty Acid Profiles of Rat Heart Sarcolemma

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The effect of various dietary fats on membrane lipid composition, fatty acid profiles and membrane-bound enzyme activities of rat cardiac sarcolemma was assessed. Four groups of male weanling Charles Foster Young rats were fed diets containing 20% of groundnut, coconut, safflower or mustard oil for 16 weeks. Cardiac sarcolemma was prepared from each group and the activities of Na^+,K^+-ATPase, 5′-nucleotidase, Ca^{2+}-ATPase and acetylcholinesterase were examined. ATPase activities were similar in all groups except the one fed coconut oil, which had the highest activities. Acetylcholinesterase activity was also similar in all the groups, however, it was significantly higher in the group fed mustard oil. No significant changes were observed among the groups in 5′-nucleotidase activity, in the cholesterol-to-phospholipid molar ratio and in sialic acid content. The coconut, safflower and mustard oil diets significantly increased cholesterol and phospholipid contents and the lipid-to-protein ratio of cardiac sarcolemma as compared to feeding the groundnut oil diet. The fatty acid composition of membrane lipids was quite different among the various groups, reflecting the type of dietary fat given. The total unsaturated-to-saturated fatty acid ratio was not different among the various groups; however, the levels of some major fatty acids such as palmitic (16:0), oleic (18:1) and linoleic (18:2) acids were significantly different. Cardiac sarcolemma of the group fed safflower oil had the highest polyunsaturated fatty acid content. The results suggest that dietary fats induce changes not only in the fatty acid composition of the component lipids but also in the activities of sarcolemmal enzymes involved in the regulation of cardiac function.
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The activities of membrane-bound enzymes are dependent on the lipid milieu surrounding the protein, and some enzymes, such as NA^+,K^+-ATPase (adenosine triphosphatase) and adenylate cyclase, have specific requirements for certain phospholipids (1). Dietary fat has been shown to affect the lipid composition of various tissues (2), including that of cardiac membranes (3), which may induce changes in the activities of membrane-bound enzymes (4). Cardiovascular health and disease are greatly dependent on the quality and quantity of dietary fat, and therefore it is of interest to study their effects at the cellular level.

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Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; CFY, Charles Foster Young; DTNB, 5,5′-dithio*bis*(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EFA, essential fatty acid; EGTA, ethyleneglycol*bis*(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PUFA, polyunsaturated fatty acids.

Na^+,K^+-ATPase and Ca^{2+}-ATPase play significant roles in the contraction and relaxation cycles of the cardiac muscle by maintaining normal ion levels within the myocytes (5). Changes in the properties of these ion pumps due to changes in the lipid microenvironment may, in turn, influence cardiac function. In fact, the failure of the cell membrane to maintain normal transmembrane ionic distribution through ion pumps is considered to be a major event in the pathogenesis of ischemia and arrythmias (6,7).

Although dietary fat is known to alter the structural lipids of various cell membranes and, in turn, to induce changes in membrane function, the exact mechanisms by which these effects are brought about are not clear. However, membrane fatty acid composition (8), cholesterol-to-phospholipid ratio (9) and optimal bilayer thickness (10) are considered important factors in bringing about these effects.

In previous studies the effect of highly saturated vegetable and animal fats (coconut oil and sheep kidney fat) on lipid composition and membrane-bound enzymes of cardiac sarcolemma has been compared to the effect of highly unsaturated dietary fats, such as safflower oil or sunflower oil (3,11–14). In the present study, we have examined the effects of several dietary vegetable oils, which markedly differ in their fatty acid composition, on the lipid composition and function of cardiac sarcolemma.

MATERIALS AND METHODS

All chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO). All solvents were of analytical grade.

Four groups of male, weanling, Charles Foster Young (CFY) rats were fed semi-purified diets containing groundnut, coconut, safflower or mustard oil at 20% by weight of the diet for 16 weeks. The composition of the diet was similar to AIN-76 A (15,16), except that the oil content was increased from 5 to 20% at the expense of starch as adapted earlier (17). Rats were killed by decapitation, hearts were removed and washed several times with ice-cold homogenizing medium containing 0.25 M sucrose, 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 M Tris-HCl buffer (pH 7.6). Cardiac sarcolemma was isolated from pooled rat hearts (n = 8–16) according to the method of Andrew and Appel (18), except that the membranes were not subjected further to sucrose density gradient centrifugation. The $105,000 \times g$ pellet was suspended in 0.25 M sucrose buffer with 10 mM Tris-HCl (pH 7.4). These membrane preparations were enriched in Na^+,K^+-ATPase and 5′-nucleotidase.

5′-Nucleotidase (EC 3.1.3.5) activity was measured according to Aronson and Touster (19) in a medium containing 100 mM glycine-NaOH buffer (pH 8.75), 3 mM sodium-AMP and 10 mM $MgCl_2$.

Na^+,K^+-ATPase (EC 3.6.1.3) activity was assayed according to Post and Sen (20), except that ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA)

was included in the reaction mixture. The reaction medium contained 50 mM Tris-HCl buffer (pH 7.4), 140 mM NaCl, 14 mM KCl, 3 mM $MgCl_2$, 3 mM Tris-ATP and 1 mM EGTA in a final volume of 0.5 mL. Ouabain (when required) was added at 1.0 mM concentration. Na^+,K^+-ATPase activity was calculated by subtracting the activity obtained with ouabain from that obtained without ouabain.

Ca^{2+}-ATPase (EC 3.6.1.3) activity was determined as described by Dhalla *et al.* (21) in a medium containing 50 mM Tris-HCl (pH 7.4) 3 mM $CaCl_2$ and 3 mM Tris-ATP. The blank contained 1 mM EGTA instead of $CaCl_2$.

Acetylcholinesterase (EC 3.1.1.8) activity was estimated according to the method of Ellman *et al.* (22), except that the reaction was stopped after 15-min incubation by the addition of an equal volume of absolute ethanol. The assay mixture contained 50 mM Tris-HCl buffer (pH 7.4), 0.2 mM Tris-EDTA, 0.1 mM 5,5′-dithio-*bis*(2-nitrobenzoic acid) and 1 mM acetylthiocholine iodide.

Lipids were extracted from the sarcolemma membrane preparations with chloroform/methanol (23). Cholesterol (24), phospholipids (25), sialic acid (26) and protein (27) were determined according to published procedures. Total lipids of cardiac sarcolemmal membranes were transesterified (28) and the fatty acids were analyzed by gas chromatography using an instrument (Model 3700, Varian Associates, Walnut Creek, CA) equipped with a flame ionization detector and an electronic integrator (Model 4270, Varian). A stainless steel column (12 ft. × 1/8 inch) was packed with 10% Silar 10C coated on Chromosorb-W, 80–100 mesh (Supelco Inc., Bellefonte, PA). Carrier gas was nitrogen at 20 mL/min. Temperature of the column was maintained at 180°C, and that of the injector and detector at 200°C. Fatty acids were identified by comparison with an authentic standard mixture (GLC-68B) obtained from Nu-Chek Prep (Elysian, MN).

All data were statistically analyzed by analysis of variance, and the level of significance was determined at $P < 0.05$.

RESULTS

The fatty acid compositions of the various dietary fats used in the experiment are shown in Table 1. Safflower oil has a very high proportion of linoleic (18:2) acid and very low amounts of saturated fatty acids. Coconut oil is characterized by a considerable proportion of short-chain fatty acids and high levels of saturated fatty acids which account for nearly 90% of total fatty acids. Mustard oil is unique in that it has erucic (22:1) acid constituting 50% of the total fatty acids. Groundnut oil has nearly 21% saturated fatty acids, 38% oleic (18:1) acid and 38% linoleic (18:2) acid. Thus all four diets differ markedly in total saturated, mono- and polyunsaturated fatty acids (PUFA).

TABLE 1

Fatty Acid Composition of Dietary Oils[a]

Fatty acid	Coconut oil	Groundnut oil	Safflower oil	Mustard oil
8:0	4.4	—[b]	—	—
10:0	6.8	—	—	—
12:0	36.7	—	—	—
14:0	26.5	—	0.1	—
16:0	10.5	12.3	2.9	2.2
18:0	3.1	4.2	2.1	1.3
18:1	9.0	38.6	15.6	10.4
18:2	3.0	38.1	79.4	15.3
18:3	—	2.2	—	14.3
20:0	—	1.8	—	—
20:1	—	—	—	3.0
22:0	—	2.8	—	—
22:1	—	—	—	53.4

[a]Values represent area % of total methyl esters.
[b]Not detectable.

The dietary fat had no influence on the cholesterol-to-phospholipid ratio; however, significant differences were observed in the absolute amounts of cholesterol and phospholipids among various groups (Table 2). Cardiac sarcolemma of safflower, mustard and coconut oil-fed groups displayed higher levels of cholesterol than did the groundnut oil-fed group. The plasma membranes of cardiac tissue of the mustard oil-fed group showed the highest levels of phospholipids, while those of the groundnut oil-fed group exhibited the lowest levels. Elevated amounts of cholesterol and phospholipids resulted in higher lipid-to-protein ratios in the cardiac sarcolemma of coconut, safflower and mustard oil-fed groups as compared to the groundnut oil-fed group. Sialic acid contents of cardiac membranes were not significantly different among the groups.

Substantial changes occurred in the fatty acid patterns of cardiac membrane lipids among the various groups (Table 3). Coconut oil diet, which was rich in saturated

TABLE 2

Lipid Composition and Sialic Acid Content of Cardiac Sarcolemma of Rats Fed Diets Containing Various Oils[a]

Dietary groups	Cholesterol (μmoles/ mgprotein)	Phospholipid phosphorus (μmoles/ mg protein)	Cholesterol/ phospholipid ratio	Lipid/protein ratio	Sialic acid (nmoles/ mg protein)
Coconut oil	0.56 ± 0.03[b]	0.86 ± 0.07[b]	0.66 ± 0.04	1.42 ± 0.10[b]	51.5 ± 7.90
Groundnut oil	0.42 ± 0.02[c]	0.64 ± 0.06[c]	0.68 ± 0.04	1.06 ± 0.08[c]	52.9 ± 7.24
Safflower oil	0.64 ± 0.04[b,d]	0.84 ± 0.07[b]	0.77 ± 0.06	1.48 ± 0.10[b]	54.4 ± 4.87
Mustard oil	0.71 ± 0.06[d]	0.99 ± 0.08[b]	0.72 ± 0.05	1.70 ± 0.13[b]	46.0 ± 5.64

[a]Values are mean ± SEM of 6–7 observations/group. Each sample was obtained by pooling 8–16 rat hearts from each group.
[b,c,d]Means in columns not sharing a common superscript are significantly different ($P < 0.05$).

TABLE 3

Total Lipid Fatty Acid Composition of Cardiac Sarcolemma from Rats Fed Diets Containing Various Oils[a]

Fatty acid	Coconut oil	Groundnut oil	Safflower oil	Mustard oil
16:0	24.2 ± 2.6[b]	29.4 ± 4.0[b]	22.1 ± 2.0[b]	16.7 ± 1.0[c]
18:0	20.4 ± 3.4	23.5 ± 5.1	25.8 ± 2.3	25.6 ± 2.7
18:1	24.4 ± 1.0[b]	22.1 ± 3.8[b]	15.7 ± 1.7[c]	20.1 ± 2.0[b]
18:2	9.0 ± 0.8[b]	8.4 ± 1.3[b]	16.8 ± 3.4[c]	11.3 ± 2.1[c]
18:3 plus 20:0	0.3 ± 0.3[b]	0.3 ± 0.3[b]	ND	1.0 ± 0.2[c]
20:1	ND[d]	0.3 ± 0.3[b]	ND	3.4 ± 0.6[c]
20:4	11.9 ± 2.8	13.0 ± 4.2	13.1 ± 1.3	7.2 ± 1.6
22:1	ND	ND	ND	3.5 ± 0.6
22:6	1.1 ± 0.8	1.2 ± 1.2	3.6 ± 1.4	4.7 ± 1.3
ΣUnsat	49.2 ± 3.2	44.9 ± 6.5	49.3 ± 4.4	53.7 ± 2.4
ΣSat	50.8 ± 3.2	54.9 ± 6.3	50.4 ± 4.6	46.3 ± 2.4
ΣMono	27.0 ± 0.6[b]	23.7 ± 2.7[b]	16.5 ± 1.4[c]	27.1 ± 0.6[b]
ΣPUFA	22.2 ± 2.8[b,c]	21.3 ± 5.5[b]	32.8 ± 2.8[c]	26.6 ± 3.0[b,c]
20:4/18:2 ratio	1.3 ± 0.3[b]	1.2 ± 0.3[c]	0.8 ± 0.2[c]	0.6 ± 0.1[c]

[a]Values are in area percentages, mean ± SEM, four samples/group; each sample was obtained by pooling 8–16 rat hearts from each group.

[b,c]Means in a horizontal row not sharing a common superscript are different ($P < 0.05$).

ΣUnsat, sum of unsaturated fatty acids; ΣSat, sum of saturated fatty acids; ΣMono, sum of monounsaturated fatty acids.

[d]Not detectable.

TABLE 4

Enzyme Activities in Cardiac Muscle Sarcolemmal Preparations from Rats Fed Different Oil Diets[a]

Enzymes	Coconut oil	Groundnut oil	Safflower oil	Mustard oil
Na^+,K^+-ATPase	61.2 ± 10.6[b]	31.6 ± 4.3[c]	30.9 ± 3.5[c]	40.4 ± 7.5[c]
Mg^{2+}-ATPase	303.7 ± 24.9[b]	210.6 ± 36.8[c]	283.8 ± 12.3[b]	208.7 ± 20.0[c]
Ca^{2+}-ATPase	313.6 ± 26.2[b]	231.7 ± 31.8[c]	286.9 ± 15.1[b]	205.0 ± 16.6[c]
Acetylcholinesterase	16.4 ± 1.2[b]	17.1 ± 0.9[b]	17.9 ± 1.9[b]	23.4 ± 1.4[c]
5′-nucleotidase	55.7 ± 5.8[b]	63.7 ± 6.0[b]	67.1 ± 10.3[b]	53.1 ± 3.9[b]

[a]All enzyme activities are in μmoles P_i/mg protein/hr, except acetylcholinesterase, which is in μmoles thiocholine/mg protein/hr. Values are mean ± SEM of 4–8 observations/group; each sample was obtained from 8–16 rat hearts from each group.

[b,c]Means in a horizontal row not sharing a common superscript are different from each other ($P < 0.05$).

fatty acids, induced in the cardiac membranes an increase in monounsaturated fatty acids, especially 18:1, and a concomitant decrease in 18:2 as compared to the safflower oil group. The safflower oil diet, which was rich in PUFA, significantly reduced the 18:1 level and consequently decreased the total monounsaturated fatty acid content. Higher levels of PUFA, especially 18:2, were found in the cardiac sarcolemma of the safflower oil-fed group than in the coconut oil-fed group.

The cardiac sarcolemma of the mustard oil-fed group was characterized by the presence of significant proportions of eicosenoic (20:1) acid and 22:1, as well as a high 18:2 level and a low palmitic (16:0) acid level. No significant differences in the arachidonic (20:4) acid level or in the unsaturated-to-saturated fatty acid ratio of the cardiac sarcolemma were observed among the groups.

Plasma membranes obtained from cardiac tissue of the four dietary groups were examined for their purity by measuring the activities of marker enzymes. The preparations were enriched with 5′-nucleotidase and Na^+,K^+-ATPase. The membrane preparations showed nearly 27–34-fold enrichment in 5′-nucleotidase activity over that of a total tissue homogenate, and the activity of this enzyme remained unaffected by dietary variation. Contamination of the sarcolemma with mitochondrial and microsomal membranes was found to be minimal as assessed by determining azide-sensitive ATPase and K^+-stimulated Ca^{2+}-ATPase (29). Furthermore, there were no differences in the yield of membrane protein among the various groups (data not shown). However, it is evident from Table 4 that the activities of other sarcolemma-bound enzymes such as Na^+,K^+-ATPase and Ca^{2+}-ATPase were elevated in the coconut oil-fed group whereas the cardiac membranes of mustard oil-fed group had the highest acetylcholinesterase activity.

DISCUSSION

Earlier studies have indicated that the lipid composition of cardiac sarcolemma is modified by including or removing certain fats (3,11–14,30) of plant or animal origin from diets. However, the effect of other commonly used

dietary lipids, such as groundnut and mustard oil, on cardiac sarcolemma was not clear until now.

Analyses of cardiac membrane lipids indicated that the various dietary fats altered the lipid-to-protein ratio and the fatty acid composition of membrane lipids, while the cholesterol-to-phospholipid ratio remained unaltered. However, increases were observed in the absolute amounts of cholesterol and phospholipids of the cardiac membranes of the coconut, safflower and mustard oil-fed groups as compared to the groundnut oil-fed group. Further, the presence of elevated levels of cholesterol in these groups suggests that the concentration of cholesterol in the cardiac membranes is independent of the PUFA content of the diet, as has been observed with liver microsomal membranes (31). Feeding fats of different fatty acid composition does not appear to alter the ratio of unsaturated-to-saturated fatty acids, suggesting that membranes generally display a considerable degree of homeostasis with respect to this parameter. This homeostasis suggests an important role for membrane lipids in regulating membrane and cellular functions.

Although no differences in the unsaturated-to-saturated fatty acid ratios were observed, the proportions of some major fatty acids of cardiac membrane lipids were significantly different between the groups. The cardiac sarcolemmal preparations from the safflower oil-fed group were characterized by higher levels of PUFA than those of the other groups. However, no significant differences were apparent in the concentration of 20:4, which may be due to the remarkable ability of cardiac tissue to conserve this fatty acid. In fact, even in severe essential fatty acid (EFA) deficiency the heart, unlike other organs, tends to maintain high levels of 20:4 (32). The higher level of PUFA in this safflower oil-fed group was mainly due to a significantly higher level of 18:2. The lower levels of PUFA (both in the groundnut and coconut oil-fed groups) were offset by concomitant increases in 18:1. Further, the cardiac membranes of the mustard oil-fed group exhibited significantly lower level of 16:0 than did those of the other three groups.

Increased PUFA content is generally thought to induce higher membrane disorder, and this trend should be apparent in the cardiac sarcolemmal membranes of safflower oil-fed group. However, in these membranes, the higher membrane disorder appears to have been compensated for by the presence of higher levels of cholesterol. Although no differences were found in the fatty acid composition of the cardiac sarcolemma between the groundnut and coconut oil-fed groups, the membranes of the latter group showed a higher lipid-to-protein ratio than those of the former group. The cardiac membranes of the coconut oil-fed group displayed higher activities of a majority of the assayed enzymes in comparison to the other groups. These observations, along with those of earlier researchers (13, 14,33–35), indicate that an optimal lipid-to-protein ratio rather than the presence of highly unsaturated fatty acids are important for the activities of many membrane-bound enzymes (33).

As judged by the activities of 5′-nucleotidase, Na^+,K^+-ATPase and cholesterol-to-phospholipid ratio, our preparations of cardiac membranes appear to be more highly purified than the sarcolemmal preparations that were used in earlier studies (35–37).

Awad and Chattopadhyay (3) reported a decrease in the activities of 5′-nucleotidase, phosphodiesterase and *p*-nitrophenylphosphatase in cardiac sarcolemma of rats fed coconut oil as compared to those of a safflower oil-fed group, and they attributed this effect to changes in the fatty acid composition of the membrane. However, the results of the present study show that the activity of 5′-nucleotidase was unaffected by dietary manipulation of membrane fatty acid composition, consistent with data reported by others (38,39).

Na^+,K^+-ATPase activity of cardiac sarcolemma was distinctly higher in the coconut oil-fed group than in the other three groups in which the enzyme showed similar activities. Increases in Na^+,K^+-ATPase activity were also reported for homogenates of salivary glands and kidneys of animals fed saturated fats, such as butter and hydrogenated coconut oil, as compared to safflower oil-fed animals (39). A similar increase in the activity of Na^+,K^+-ATPase has been reported for liver plasma membranes (40) and brain synaptosomes (41) of EFA-deficient animals. The activities of Mg^{2+}-ATPase and Ca^{2+}-ATPase of sarcolemma were higher in the coconut oil and safflower oil-fed groups than in the other two groups. Surprisingly, two dietary fats with diverse fatty acid compositions exerted similar stimulatory effects on the activities of these two enzymes. The mechanisms by which these effects on membrane-bound enzyme activities are brought about are not clearly understood. Enhanced enzyme activities have been attributed to an increase in enzyme protein (42), changes in membrane bilayer thickness (43) and changes in lipid-to-protein ratio (33,44,45). Further studies are required to cast more light on the influence of various dietary fats on these membrane parameters.

The enhanced activity of acetylcholinesterase in the mustard oil-fed group may result in decreased levels of acetylcholine which would affect neuromuscular function. The importance of surface phenomena (46) and the role of sialic acid (47,48) in determining the activity of this enzyme have been recognized. The insertion of membrane proteins into the lipid core may be dependent on the transmembrane potential (49,50), which could be maintained by sialic acid by virtue of its negative charge (48). However, no differences in sialic acid content were found in the cardiac membranes of the groups, thereby suggesting the existence of other regulatory mechanisms.

The results of the present study suggest that the impact of dietary fats on the activities of membrane-bound enzymes can be mediated through factors such as fatty acyl composition of the membrane lipids and lipid-to-protein ratio of the membrane.

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Effect of Prenatal and Postnatal Exposure to Ethanol on Rat Central Nervous System Gangliosides and Glycosidases

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We investigated the effect of maternal alcohol consumption on cell number, gangliosides and ganglioside catabolizing enzymes in the central nervous system (CNS) of the offspring. Virgin female rats of the Charles Foster strain were given 15% (v/v) ethanol in drinking water one month prior to conception and during gestation and lactation. At 21 days postnatal age, the offspring were sacrificed and the brains were separated into cerebrum, cerebellum and brain stem to investigate possible regional variations. Compared to controls, wet weight of cerebrum, cerebellum and brain stem, and of spinal cord was decreased in the pups exposed to alcohol. DNA and protein contents were also found to be lowered in all the CNS regions of the pups exposed to alcohol. Conversely, maternal alcohol consumption was found to increase the concentration and the content of total ganglioside *N*-acetylneuraminic (NANA) in CNS of the pups. In addition, alcohol treatment was found to induce alterations in the proportions of individual ganglioside fractions. Interestingly, these alterations are somewhat different than those observed in the neonatal brain and spinal cord of the pups subjected to prenatal alcohol exposure. The alterations in the proportions of ganglioside fractions were shown to be region-specific. Maternal alcohol consumption resulted in decreased activities of sialidase, β-galactosidase, β-glucosidase and β-hexosaminidase. The results suggest that the alcohol-associated increases in ganglioside concentration may be at least partly due to the decreased activities of ganglioside catabolizing enzymes.
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Alcohol consumption during pregnancy is detrimental to the fetus, and the nervous system has been shown to be affected most (1). Clusters of abnormalities observed in children exposed to alcohol *in utero* are described as fetal alcohol syndrome (FAS). The complete spectrum of abnormalities of FAS includes pre- and postnatal growth retardation, microcephaly, facial dysmorphology and central nervous system (CNS) dysfunction (2,3). To study the effects of alcohol on rat brain development, it is necessary to expose the offspring to alcohol during both pre- and postnatal periods, as rats, unlike humans, are born relatively immature with respect to their brain development (4).

The plasma membrane appears to be the primary target of the effects of alcohol; hence much research in this area has been focused on lipids, which are major constituents of neural membranes. However, most studies have been confined to cholesterol and phospholipids, which are important for maintaining membrane integrity and fluidity. Neural membranes (5), particularly those of brain, also are rich in gangliosides (6) which play an important role in a wide variety of cellular events, ranging from cell differentiation (7) to neuronal transmission (8). Despite their suggested importance, information on the effects of alcohol on CNS gangliosides is scanty and is mostly restricted to adult rats. There are no studies on the effects of pre- and postnatal alcohol exposure on the enzymes involved in the catabolism of gangliosides.

We have reported the effects of prenatal exposure to alcohol on rat brain gangliosides at birth. However, growth spurt in rat brain development (9) and the rapid phase of accumulation of gangliosides (10) occur during the postnatal period. As the brain is a heterogeneous organ, the present study was designed to follow the effects of pre- and postnatal exposure to alcohol on the cell number and protein content, and on gangliosides and their catabolizing enzymes in cerebrum, cerebellum, brain stem and spinal cord of pups at weaning.

MATERIALS AND METHODS

Experimental design and animals. Virgin female rats of the Charles Foster strain weighing 180–200 g were divided into two groups. A control group (CT) was given a 20% casein diet and drinking water *ad libitum,* and a second group of rats (AL) was given 20% casein diet and 15% alcohol (ethanol) in drinking water *ad libitum.* The rats were fed a semi-synthetic diet containing 20% sucrose, 20% casein, 7% ground nut oil (peanut oil), 0.3% DL-methionine, 0.2% choline bitartrate, 48% sago, 3.5% mineral mixture, and vitamin mixture (11). Both groups of rats were maintained on the respective regimen for four weeks prior to breeding and during gestation and lactation. After birth, pups were culled randomly and nursed in litters of eight. Pups were sacrificed by decapitation, at 21 days postnatal age, after the body weights had been recorded. Intact whole brains and spinal cords were quickly dissected and the weights were recorded. Whole brains were separated into cerebrum, cerebellum and brain stem on ice-cold glass plates. Tissue samples for DNA, protein and enzyme assays were processed immediately; the samples for ganglioside analysis were stored until use in a chloroform/methanol (1:1, v/v) mixture at −20°C.

Estimation of maternal plasma alcohol levels. Tail blood samples from the mothers were collected in the morning after birth. Alcohol was assayed using alcohol dehydrogenase (12). Blood ethanol concentration was found to be 87.3 ± 10 mg/dL (mean ± SD).

Estimation of DNA and protein. DNA was extracted by the method of Schneider (13) and was estimated according to the method of Burton (14). Protein was estimated by the method of Lowry *et al.* (15) using bovine serum albumin as standard.

Analysis of gangliosides. Total gangliosides were isolated and purified by the method of Seyfried *et al.* (16), and ganglioside *N*-acetylneuraminic acid (NANA) was assayed by the method of Warren (17) as modified by

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Abbreviations: AL, pups exposed to alcohol; BS, brainstem; C, cerebrum; CB, cerebellum; CNS, central nervous system; CT, control pups; FAS, fetal alcohol syndrome; NANA, *N*-acetylneuraminic acid; SC, spinal cord.

Skoza and Mohos (18). The details of these methods are described elsewhere (19). The ganglioside nomenclature used is that of Svennerholm (20). For the separation of individual gangliosides (GQ_1, GD_{1b}, GD_{1a}, GT_{1b} and GM), 20 × 20-cm glass plates coated with silica gel G were developed by one ascending run with chloroform/methanol/water containing 0.02% $CaCl_2 \times 2H_2O$ (58:38:9, v/v/v). Ganglioside bands marked after exposure to iodine vapors were scraped from the plates and NANA in the individual ganglioside fractions was estimated as described earlier. Since the concentration of monosialogangliosides such as GM_2 and GM_3 was extremely low, the bands corresponding to all monosialogangliosides were pooled and the fraction was designated GM. The bands were confirmed as gangliosides by using resorcinol-HCl reagent on a duplicate set, and the identity of individual ganglioside bands was confirmed by simultaneously running a commercially obtained reference bovine brain ganglioside mixture (Sigma, St. Louis, MO) (19).

Enzyme assays. A 10% (w/v) homogenate for enzyme assays was prepared by homogenizing the tissue with ice-cold, glass-distilled water for three minutes at 2000–3000 rpm using a Potter-Elvehjem homogenizer while cooling with ice. Activities of β-D-galactosidase (EC 3.2.1.23) and β-D-glucosidase (EC 3.2.1.21) were assayed according to the method of Gatt and Rapport (21). Total β-D-hexosaminidase (EC 3.2.1.30) activity was assayed according to the method of Frohwein and Gatt (22). Units for the abovementioned enzymes are defined as nmol of *p*-nitrophenol liberated per minute under the assay conditions used. Sialidase (EC 3.2.1.18) activity, using endogenous substrate alone, was assayed according to the method of Irwin *et al.* (23). The enzyme unit for sialidase is defined as nmol of NANA liberated per minute under assay conditions. Statistical comparison between group means was by Student's *t*-test (24). Differences between values were considered significant for $p < 0.01$.

RESULTS

In preliminary studies we found that providing a 15% (v/v) alcohol solution as the sole water source had no effect on the total calorie or water intake of rats (data not shown). However, higher concentrations of alcohol did cause a significant decrease in food and water intake. The data obtained for tissue weights, and DNA and protein levels in the CNS regions of 21-day-old pups exposed to alcohol *in utero* and during lactation are presented in Table 1. Pre- and postnatal exposure of rat pups to alcohol caused significant deficits in cerebrum, cerebellum, brain stem and spinal cord weights as compared to controls. The tissue weight loss was more severe in brain stem followed by cerebellum and cerebrum, in that order. The deficits in tissue weights were 11, 15, 29 and 16% in cerebrum, cerebellum, brain stem and spinal cord, respectively.

DNA content (mg DNA/total tissue) showed a significant decrease in all CNS tissues of the pups exposed to alcohol when compared to the control pups. DNA content in cerebrum, cerebellum, brain stem and spinal cord of the alcoholic pups was 84, 88, 83 and 66% of control values, respectively. These deficits in DNA contents were reflected in the reduced cell number. The reductions in cell numbers of pups exposed to alcohol were 16, 12, 17 and 34% of controls in cerebrum, cerebellum, brain stem and spinal cord, respectively. Similarly, protein content (mg/total tissue) showed a significant decrease in all CNS regions.

TABLE 1

Effect of Alcohol on DNA and Protein Levels in the Central Nervous System[a]

Tissue	Group	Tissue weight (g)	DNA		Protein		Cell number (in millions)
			mg/g tissue	mg/tissue	mg/g tissue	mg/tissue	
C	CT (n=6)	1.098 ±0.006	1.85 ±0.007	2.03 ±0.02	123 ±0.33	135 ±0.99	327 ±4.0
	AL (n=6)	0.980[b] ±0.002	1.730 ±0.02	1.70[b] ±0.02	121 ±0.35	118[b] ±0.42	274[b] ±3.2
CB	CT (n=6)	0.186 ±0.002	9.2 ±0.04	1.7 ±0.016	104 ±0.4	19.3 ±0.19	244 ±2.4
	AL (n=6)	0.159[b] ±0.001	9.50[b] ±0.06	1.50[b] ±0.014	101 ±0.22	16.2[b] ±0.05	227[b] ±3.0
BS	CT (n=6)	0.153 ±0.012	2.14 ±0.043	0.326 ±0.005	104 ±1.0	15.8 ±0.12	52.6 ±0.84
	AL (n=6)	0.108[b] ±0.002	2.47[b] ±0.037	0.270[b] ±0.005	100 ±0.07	11.0[b] ±0.17	43.5[b] ±0.8
SC	CT (n=6)	0.182 ±0.03	2.52 ±0.04	0.458 ±0.008	100 ±0.67	18.3 ±0.23	73.9 ±1.31
	AL	0.153[b] ±0.001	1.98[b] ±0.02	0.304[b] ±0.003	101 ±0.4	15.4[b] ±0.1	49.0[b] ±0.5

[a]CT, control pups born of mothers fed a 20% casein diet. AL, alcoholic pups born of mothers fed 15% alcohol; C, cerebrum; CB, cerebellum; BS, brainstem; SC, spinal cord; n, number of observations; values are mean ± SE.

[b]Values significantly different from the CT group if value of $p < 0.01$.

TABLE 2

Effect of Alcohol on CNS Gangliosides at Weaning[a]

Tissue	Group	Total gangliosides		Ganglioside species (% of total ganglioside NANA)				
		μg NANA/g tissue	μg NANA/tissue	GQ_1	GT_{1b}	GD_{1b}	GD_{1a}	GM
C	CT (n=8)	706 ±4.6	791 ±11.8	9.9 ±0.4	21.1 ±0.32	14.1 ±0.37	43.8 ±0.49	11.1 ±0.49
	AL (n=6)	954[b] +17	924[b] ±14	4.1[b] ±0.5	29.4[b] ±0.3	14.4 ±0.56	38.2[b] ±0.19	14.0[b] ±0.30
CB	CT (n=8)	398 ±6.5	71.1 ±1.5	6.7 ±0.5	44.3 ±0.2	10.3 ±0.3	26.5 ±0.39	12.5 ±0.38
	AL (n=8)	587[b] ±14	93.8[b] ±1.5	8.9[b] ±0.3	36.0[b] ±0.2	13.8[b] ±0.3	24.6 ±0.37	16.5[b] ±0.3
BS	CT (n=8)	395 ±13	59.6 ±2	3.3 ±0.3	34.7 ±0.3	22.1 ±0.7	22 ±1	17.8 ±0.7
	AL (n=6)	635[b] ±25	70.9[b] ±3	7.4[b] ±0.5	42.3[b] ±0.67	21.4 ±0.26	20.1 ±0.41	8.8[b] ±0.4
SC	CT (n=8)	291 ±4	56 ±1	18.6 ±0.2	31.6 ±0.8	13.3 ±0.6	22 ±0.4	14.6 ±0.5
	AL (n=8)	371[b] ±11	57.5 ±1.2	15.0[b] ±0.4	29.6 ±0.4	17.5[b] ±0.4	27.6[b] ±0.6	10.0[b] ±0.3

[a]CT, control pups born of mothers fed a 20% casein diet; AL, alcoholic pups born of mothers fed 15% alcohol; C, cerebrum; CB, cerebellum; BS, brain stem; SC, spinal cord; n, number of observations; values are mean ± SE.

[b]Values significantly different from the CT group if value of $p < 0.01$.

Administration of alcohol to rats during gestation and lactation caused an increase in total ganglioside NANA concentration (μg NANA/g tissue) in cerebrum, cerebellum, brain stem and spinal cord of the pups when compared to controls (Table 2). The increase in total ganglioside concentration was 35, 47, 61 and 27%, respectively, in cerebrum, cerebellum, brain stem and spinal cord, compared to control values. Despite reduced tissue weights, the content of total ganglioside NANA (μg NANA/total tissue) was significantly higher in cerebrum, cerebellum and brain stem of alcoholic pups when compared to controls.

Alcohol was also found to induce alterations in the proportions of individual ganglioside fractions, and the changes were found to be region-specific. In general, alcohol-induced alterations in the proportions of GQ_1, GT_{1b} and GD_{1a} were more pronounced. In cerebrum, the proportions of GT_{1b} and GM showed an increase, GQ_1 and GD_{1a} showed a decrease, and GD_{1b} was unaffected by exposure of pups to alcohol. On the other hand, in cerebellum, alcohol increased the proportions of GQ_1, GD_{1b} and GM and decreased the proportion of GT_{1b} without affecting the proportions of GD_{1a}. However, in brain stem the proportions of GQ_1 and GT_{1b} showed a significant increase and GM showed a decrease in pups exposed to alcohol. The proportions of GD_{1a} and GD_{1b}, which were unaltered in brain stem, showed an increase in the spinal cord of the alcoholic pups. However, the proportions of GQ_1 and GM showed a decrease in spinal cord due to alcohol.

Sialidase, β-glucosidase, β-galactosidase, pH 3.1 and β-hexosaminidase activities decreased in cerebrum, cerebellum, brain stem and spinal cord of the pups due to maternal alcohol consumption (Table 3). A similar decrease was observed in the β-galactosidase, pH 4.5 activity in cerebrum, cerebellum and brain stem following exposure to alcohol. However, alcohol was found to increase β-galactosidase, pH 4.5 activity in the spinal cord of alcoholic pups as compared to control pups. Sialidase, β-hexosaminidase, β-glucosidase, β-galactosidase, pH 3.1 and β-galactosidase, pH 4.5 activities, in cerebrum of alcohol exposed pups were only 65, 70, 69, 91, and 89% of control values. The corresponding values in cerebellum, brain stem and spinal cord were 78, 85, 75, 84 and 94%; 66, 61, 75, 78 and 77%; and 70, 88, 82, 93 and 77%, respectively.

DISCUSSION

In the present study rat pups were exposed to alcohol by feeding mothers with 15% alcohol one month before gestation, and during gestation and lactation. Alcohol consumed by the mother during gestation reaches the fetus as there is no placental barrier to alcohol (25); after birth, pups were exposed to alcohol through the mother's milk (26).

Alcohol-associated deficits in DNA content observed in the present study correlate well with the reported decrease in the incorporation of labeled thymidine into fetal tissues (27) and with impaired cellular multiplication observed *in vitro* due to alcohol (28). Alcohol also was found to decrease the levels of zinc and folate (29,30), which are required for DNA synthesis. Taken together the studies suggest that alcohol-associated cell loss may be due to

TABLE 3

Effect of Alcohol on Rat CNS Ganglioside Catabolizing Enzymes at Weaning[a]

Tissue	Group	β-D-Galactosidase[c] Activity at pH 3.1	β-D-Galactosidase[c] Activity at pH 4.5	β-D-Glucosidase[c]	β-D-Hexosaminidase[c]	Sialidase[c]
C	CT (n=10)	222 ±0.9	128 ±0.5	210 ±4.5	2930 ±32	13.1 ±0.41
	AL (n=10)	202[b] ±3.0	114[b] ±1.5	145[b] ±4.5	2053[b] ±64	8.5[b] ±0.78
CB	CT (n=10)	422 ±4.4	219 ±1.7	137 ±2	2897 ±39	7.9 ±0.78
	Al (n=10)	356[b] ±6.5	206[b] ±4.0	92[b] ±2.5	2460[b] ±46	6.2 ±0.61
BS	CT (n=10)	388 ±5.7	225 ±2.5	171 ±3.1	2695 ±60	8.9 ±0.14
	AL (n=10)	301[b] ±3.7	173[b] ±2.7	128[b] ±1.8	1652[b] ±43	5.9[b] ±0.11
SC	CT (n=10)	330 ±7.3	155 ±2.9	184 ±3.2	1985 ±39	8.1 ±0.76
	UN (n=10)	306[b] ±5.5	168[b] ±2.0	150[b] ±1.4	1742[b] ±43	5.7[b] ±0.36

[a] CT, control pups born of mothers fed a 20% casein diet; AL, alcoholic pups born of mothers fed 15% alcohol; C, cerebrum; CB, cerebellum; BS, brain stem; SC, spinal cord; n, number of observations; values are mean ± SE.

[b] Values significantly different from the CT group if value of $p < 0.01$.

[c] Enzyme units/g tissue.

impairment in DNA synthesis. Postnatal alcohol exposure has been shown to induce neuronal cell loss in hippocampus and cerebellum (31) and to delay the maturation of oligodendrocytes (32). Deficits observed in CNS cell numbers of the alcoholic pups are in line with a 29% cell loss in the cerebellum reported by Burns *et al.* (33) following the administration of alcohol (4 g/Kg body weight) between 6-16 days postnatal. In the present study, the cell loss was found to be 16, 12, 17 and 34% in cerebrum, cerebellum, brainstem and spinal cord, respectively, when compared to the controls. Alcohol also was found to decrease the protein content. This loss in protein content could be attributed to alcohol related impairment in the uptake of amino acids across the placenta (34,35) and in protein synthesis (36).

In contrast, the concentration of total ganglioside NANA (μg NANA/g tissue) and content (μg NANA/total tissue) was higher in all brain regions following exposure to alcohol. This increase in ganglioside concentration is in agreement with the reported increase in adult mouse (37) and rat (38) brain ganglioside concentration following chronic alcohol administration. In this context it may also be relevant to note that chronic alcohol treatment increases the intracellular fluid volume (39,40) and that maternal alcohol consumption increases water and sodium content in the fetus (41) and increases brain cell size of the offspring (33).

Although alcohol tends to fluidize biological membranes, chronic exposure to alcohol increases order in membranes by increasing the concentration of cholesterol (42). It is believed that the increase in cholesterol content, following chronic exposure to alcohol, is to confer rigidity to the membranes to counteract the fluidizing effects of alcohol. An increase in gangliosides has been shown to reduce the integrity of lipid bilayers (43) and to increase the sensitivity of liposomes to alcohol (44). Alcohol also was found to increase the content of unsaturated fatty acids of phospholipids in the synaptosomal fraction (45). Such an increase in the unsaturated fatty acids would also increase membrane fluidity.

It is of interest to note that the alcohol induced alterations in the proportions of individual gangliosides that were observed in the present study are somewhat different than the alterations observed in the neonatal pups subjected to alcohol exposure *in utero* (19). In addition, the changes in the ganglioside proportions are region-specific. For example, trisialoganglioside GT_{1b} showed an increase in cerebrum and brain stem and a decrease in cerebellum following exposure to alcohol. On the other hand, disialoganglioside GD_{1a} showed a decrease in cerebrum and an increase in spinal cord due to alcohol. Polysialoganglioside GQ_1 showed a decrease in cerebrum and spinal cord and an increase in cerebellum and brain stem of pups exposed to alcohol. Monosialogangliosides (GM) showed an increase in cerebrum and a decrease in the myelin rich regions, such as brain stem and spinal cord, due to alcohol. This decrease in monosialoganglioside (GM) is consistent with the reported decrease in monosialoganglioside GM_1 in myelin of 24-day-old rat pups born of mothers fed 6.6% (v/v) ethanol diet prior to breeding and during gestation (46). In addition, postnatal alcohol exposure was found to delay the acquisition of myelin by rat optic nerve (47).

In conclusion, it would appear that the changes in gangliosides observed can have profound implications in various surface events, such as cell differentiation, cell-to-cell interaction, and synaptogenesis, as gangliosides are located on the outer layer of the plasma membrane with their negatively charged polysaccharide chains protruding towards the extracellular space.

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Reaction of Dipyridamole with the Hydroxyl Radical

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Dipyridamole [2,6-*bis*-diethanolamino-4,8-dipiperidinopyrimido-(5,4-d)pyrimidine], a well known platelet aggregation inhibitor, shows powerful hydroxyl radical scavenging activity by inhibiting OH•-dependent salicylate and deoxyribose degradation. Steady-state competition kinetics experiments with deoxyribose were carried out to evaluate the second-order rate constant for the reaction between hydroxyl radical and dipyridamole. OH• radicals were generated either by a Fenton-type reaction or by X-ray irradiation of water solutions. A second-order rate constant $k_{(Dipyridamole + OH\cdot)}$ of $1.72 \pm 0.11 \times 10^{10}$ M^{-1} s^{-1} and of $1.54 \pm 0.15 \times 10^{10}$ M^{-1} s^{-1} was measured by Fenton chemistry and by radiation chemistry, respectively. Mannitol was used as an internal standard for hydroxyl radicals in steady-state competition experiments with deoxyribose. A rate constant $k_{(Mannitol + OH\cdot)}$ of $1.58 \pm 0.13 \times 10^9$ M^{-1} s^{-1} and $1.88 \pm 0.14 \times 10^9$ M^{-1} s^{-1} was measured in the Fenton model and in the water radiolysis system, respectively. Both these rate constants are in good agreement with the published data obtained by the "deoxyribose assay" and by pulse radiolysis.
Lipids 27, 349–353 (1992).

There is a large amount of data which support the involvement of oxygen free radicals (OFR) in various human diseases and in aging (1). One of the most important OFR species is the highly reactive hydroxyl radical, which causes tissue injury through a variety of pathways including the initiation of lipid peroxidation, DNA damage and inactivation of proteins. The use of antioxidants to prevent the deleterious effects of OFR could be one approach in the therapy of several of these diseases. Consistent with this, a number of therapeutic agents may actually exert some of their beneficial effects by scavenging OFR (2–5).

Dipyridamole (Fig. 1) is a drug that is currently used in the treatment of cardiovascular diseases because of its vasodilating activity and its ability to inhibit platelet aggregation. Dipyridamole also has been shown to inhibit lipid peroxidation (6,7) and to scavenge OFR (7). A better understanding of the antioxidant activity of dipyridamole will be useful to elucidate its pharmacological action.

This study was designed to investigate the reaction of dipyridamole with the hydroxyl radical. Measurements involving this radical are complicated because of its high reactivity and its participation in a multitude of reactions. It is therefore useful to employ complementary techniques to examine the same reaction. To study the interaction between OH• and dipyridamole, two types of assays were used. In the first, hydroxyl radicals were generated in aqueous solution *via* an ascorbate-modified Fenton reaction where the metal catalyst was bound to EDTA to produce "free" OH• in solution (8). In the second system, OH• radicals were generated by radiation-induced decomposition of water. To further evaluate the results, control experiments were carried out in the presence of the well-characterized hydroxyl radical scavenger mannitol.

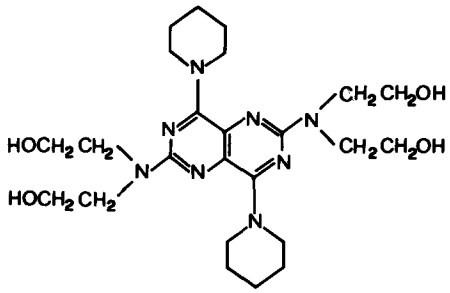

FIG. 1. Structure of dipyridamole.

MATERIALS AND METHODS

Dipyridamole was donated by Boehringer Ingelheim Italia (Reggello, Italy). Deoxyribose, salicylate, 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate and 3,4-dihydroxybenzoate were from Aldrich (Steinheim, Germany). High-performance liquid chromatography (HPLC)-grade solvents were all purchased from Carlo Erba (Rome, Italy). All other reagents were of the highest grade available from Merck (Darmstadt, Germany). Water of high purity (≥18 MOhms) was obtained by treating doubly distilled water in a Milli-Q (Millipore, Bedford, MA) purifying system.

OH• production by Fenton-like chemistry. Incubations for 1 hr at 37 °C were carried out in Falcon polypropylene sterile-tubes (Becton-Dickinson, Milan, Italy). The reaction mixtures contained, in a final volume of 1 mL, the reagents (added in the following order): KH_2PO_4/KOH buffer (20 mM),deoxyribose (2.8 mM), EDTA (104 μM), $FeCl_3$ (100 μM), H_2O_2 (1 mM), and ascorbate (100 μM). The pH was 7 for mannitol experiments and 5 for experiments with dipyridamole, because dipyridamole is insoluble above pH 5.5. Stock solutions of Fe^{3+}, H_2O_2 and ascorbate were made up in water immediately before use.

OH• production by water radiolysis. Irradiation was with X-rays in an atmosphere of air. The solutions contained 2.8 mM deoxyribose and 10 mM potassium phosphate as buffer in Falcon polystyrene-sterile tubes. The tubes were placed in the X-ray source (Stabilipan, Siemens, Erlangen, Germany) at 22 °C. The dose rate (11.3 Gy/min) was determined by Fricke dosimetry (9). Most irradiation treatments lasted 20 min. At the end of the irradiation period, reaction mixtures were treated as described above for the determination of malonaldehyde derived from the OH•attack on deoxyribose.

Measurement of OH•-dependent deoxyribose degradation. To the reaction mixture was added 1 mL of 2.8% trichloroacetic acid plus 1 mL of thiobarbituric acid (1% w/v in 50 mM NaOH) and the mixture was heated for 20

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; OFR, oxygen free radicals; TBA, thiobarbituric acid.

min at 100°C. The chromogen was measured at 532 nm on an HP 8542A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA).

Measurement of OH•-dependent salicylate oxidation. At the end of the irradiation time, 500 μL of each sample was placed on ice and 10 μL of 3,4-dihydroxybenzoic acid (internal standard) plus 25 μL of 1 M HCl were added, and the mixture was vortexed. The resulting solution was extracted with 5 mL of HPLC grade diethyl ether by mixing thoroughly for 1 min and then centrifuged for 5 min at 2,000 × *g*. The organic phase was collected, evaporated to dryness under nitrogen, and reconstituted with 2.0 mL of HPLC mobile phase. The samples were injected for HPLC analysis immediately after reconstitution.

Reverse phase HPLC separations were carried out on a 5 ODS Spherisorb 250 mm × 4.6 mm i.d. analytical column, using a Perkin Elmer series 410 LC pump equipped with a Perkin Elmer SEC-4 solvent environmental control (Perkin Elmer Co., Norwalk, CT). The mobile phase consisted of 90% (v/v) 30 mM sodium citrate, 27.7 mM acetate buffer (pH 4.75) and 10% (v/v) methanol, according to Grootveld and Halliwell (10). The mobile phase was filtered through a Millipore filter of 0.45 μm pore size and continuously purged with He gas during elution. The flow rate was 1 mL/min; the injection loop was 50 μL. The column eluate was monitored with an LC4B amperometric detector (Bioanalytical Systems, West Lafayette, IN) equipped with a Bioanalytical Systems detector cell. The detector potential was set at +0.76 V *vs.* an Ag/AgCl reference electrode. The output of the detector was registered on an Isco Chemresearch Data Management System (Lincoln, NE). The range was set at 10 nA. Concentrations were determined from calibrations with standard solutions of 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate, using 3,4-dihydroxybenzoate as internal standard. The retention times relative to salicylate (12 ± 0.4 min) were 0.45, 0.49 and 0.66 for 2,3-dihydroxybenzoate, 2,5-dihydroxybenzoate and 3,4-dihydroxybenzoate, respectively.

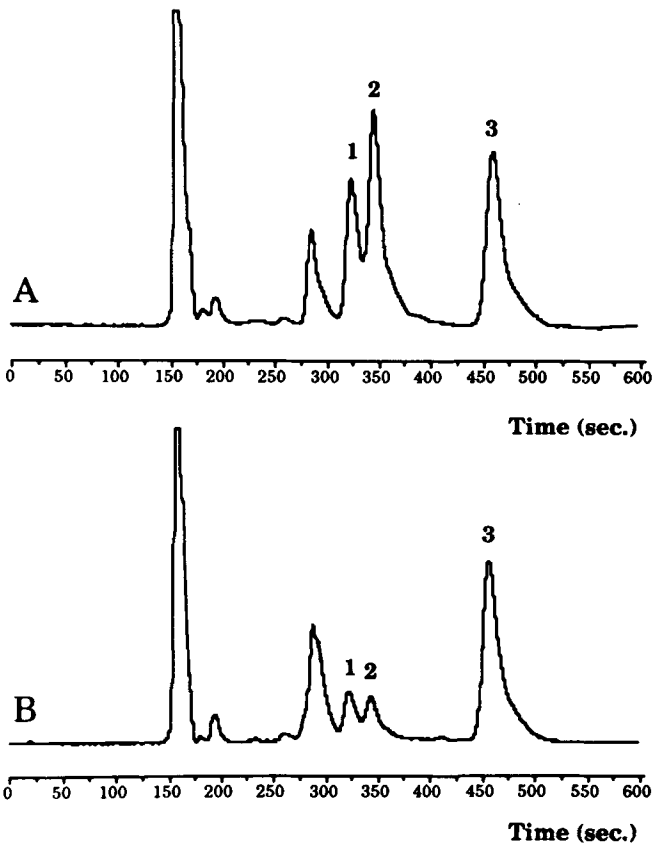

FIG. 2. Inhibition of OH•-dependent salicylate oxidation by dipyridamole. HPLC analysis of 10 μM salicylate after exposure to OH•, in the absence (A) or presence (B) of dipyridamole (100 μM). Peak identification: 1, 2,3-dihydroxybenzoate; 2, 2,5-dihydroxybenzoate; and 3, 3,4-dihydroxybenzoate (internal standard).

RESULTS

The reaction of dipyridamole with OH• was monitored by following the inhibition of OH•-dependent salicylate oxidation in the water radiolysis system. OH• attacks the salicylate molecule and gives addition products measured by reverse phase HPLC:

$$OH\bullet + \text{salicylate} \rightarrow \text{dihydroxybenzoates} \quad [1]$$

Figure 2, upper panel, shows the chromatographic profile of 10 μM salicylate after exposure to OH•, the 2,3- and 2,5-dihydroxybenzoates generated were identified as peaks 1 and 2, respectively. The lower panel of Figure 1 shows the same experiment carried out in the presence of dipyridamole, which lowers the amounts of 2,3- and 3,5-dihydroxybenzoates formed according to reaction [2].

$$OH\bullet + \text{dipyridamole} \rightarrow \text{products} \quad [2]$$

Neither dipyridamole nor its oxidation product could be detected with the HPLC column/solvent combination, and the exact nature of the reaction product is still unknown. However, product formation was indicated through observed changes in the optical spectrum of dipyridamole (Figs. 3 and 4); the characteristic absorption maximum at 404 nm disappeared gradually upon exposure to OH•. The existence of an isosbestic point suggested that a single product was formed during the reaction. Addition of the hydroxyl radical scavenger sodium formiate slowed down the dipyridamole degradation considerably, consistent with the involvement of OH• in the process (Fig. 4). In a series of parallel experiments it was found that dipyridamole also inhibited salicylate oxidation in the Fenton system (not shown).

The magnitude of the OH• scavenging activity of dipyridamole was investigated by steady-state competition kinetics experiments in order to measure the rate constant of reaction [2]. Deoxyribose was used in competition kinetic experiments as reference substance:

$$OH\bullet + \text{deoxyribose} \rightarrow \text{malondialdehyde} \quad [3]$$

The use of deoxyribose as detector in competition experiments was proposed by Halliwell *et al.* (8) and has proved a reliable alternative to pulse radiolysis. The amount of malonaldehyde generated (11) in reaction [3] was measured by the thiobarbituric acid (TBA) test.

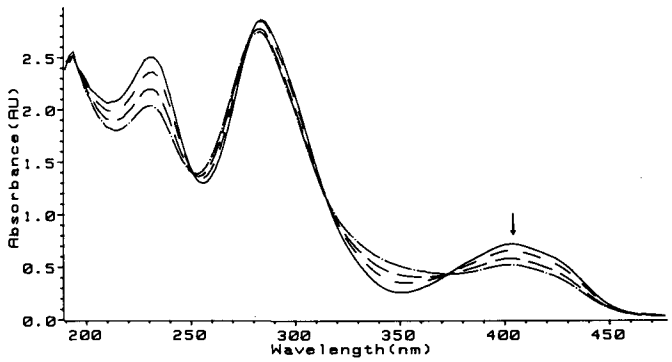

FIG. 3. Changes of the dipyridamole spectrum upon increasing exposition to OH•. Dipyridamole (100 μM) was irradiated at a rate of 11.3 Gy/min in 10 mM phosphate, pH 5, 22°C, in air atmosphere.

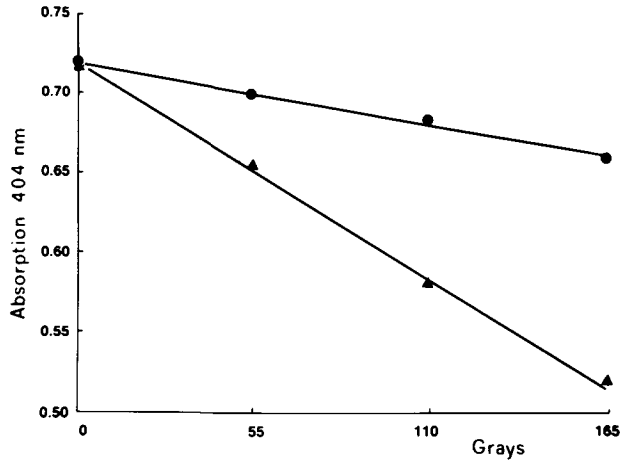

FIG. 4. Time course of absorbance loss (404 nm), in the absence (triangles) or presence (circles) of 100 mM sodium formiate, as hydroxyl radical scavenger. Experimental conditions as in Figure 3.

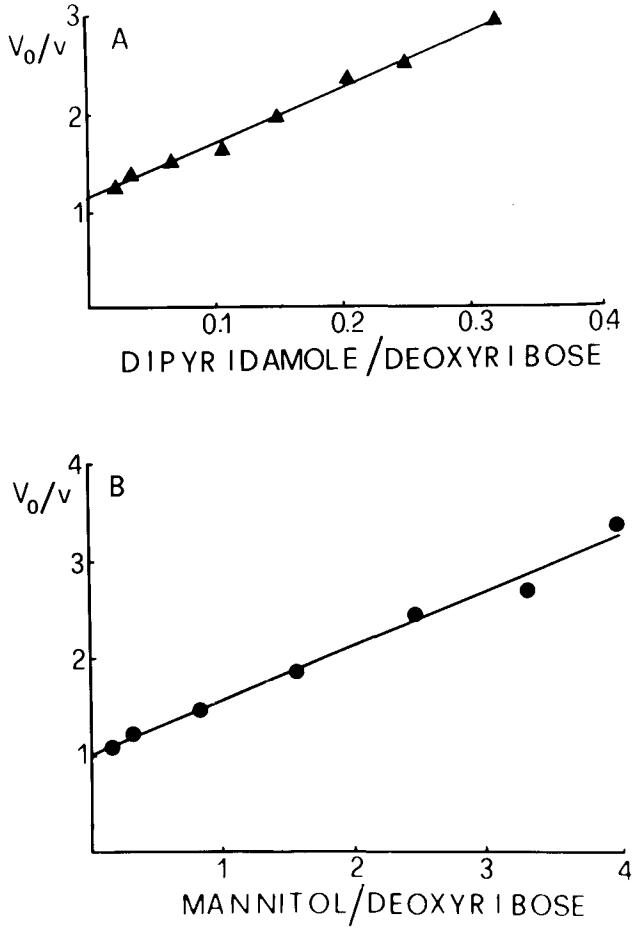

FIG. 5. Competition plots of dipyridamole (plot A) or mannitol (plot B), and deoxyribose with a Fenton-type reaction as OH• source. Concentration of deoxyribose was 2.8 mM, pH 5 for dipyridamole, pH 7 for mannitol. See Materials and Methods for details. Each point represents the mean of three separate experiments carried out in triplicate, error bars are not shown as they do not exceed the size of the symbols.

The rate of the absorption change (V_0) of deoxyribose, in the absence of competitor molecules, was taken as a measure of the rate of reaction, which is given by:

$$V_0 = k_3[\text{deoxyribose}][OH\bullet]$$

In the presence of different concentrations of the competitor dipyridamole, the rate of reaction (v) is:

$$v = V_0 \frac{k_3\,[\text{deoxyribose}]}{k_3\,[\text{deoxyribose}] + k_2\,[\text{dipyridamole}]}$$

A minor transformation yields a linear equation:

$$\frac{V_o}{v} = 1 + \frac{k_2}{k_3}\,\frac{[\text{dipyridamole}]}{[\text{deoxyribose}]}$$

Plotting V_0/v against [dipyridamole]/[deoxyribose] gives a straight line intersecting the ordinate at unity. The rate constant for the reaction of dipyridamole with OH• can be obtained from the slope assuming a k_3 of 3.1×10^9 M^{-1} s^{-1} (8). Figure 5 shows competition experiments with deoxyribose carried out in the Fenton chemistry system. Plot A refers to dipyridamole; from the slope a $k_{(\text{Dipyridamole} + OH\cdot)}$ of 1.72×10^{10} M^{-1} s^{-1} was calculated (mean value). Figure 6 summarizes the data of analogous experiments which were carried out with irradiation generated OH•. Also in this case, the data from experiments carried out in the presence of dipyridamole (plot A) give a straight line, from which a rate constant of 1.54×10^{10} M^{-1} s^{-1} was calculated (mean value).

Competition experiments were also carried out with mannitol as internal standard since the bimolecular rate constant for the reaction of mannitol with OH• is known from pulse radiolysis measurements. We obtained a $k_{(\text{Mannitol} + OH\cdot)}$ of 1.58×10^9 M^{-1} s^{-1} (Fig. 5B) and 1.88×10^9 M^{-1} s^{-1} (Fig. 6B); these values (Table 1) are very similar to the published rate constants obtained by the "deoxyribose assay" and by pulse radiolysis (8,12).

DISCUSSION

It has been reported that dipyridamole scavenges OH• in experiments using deoxyribose as detector molecule for

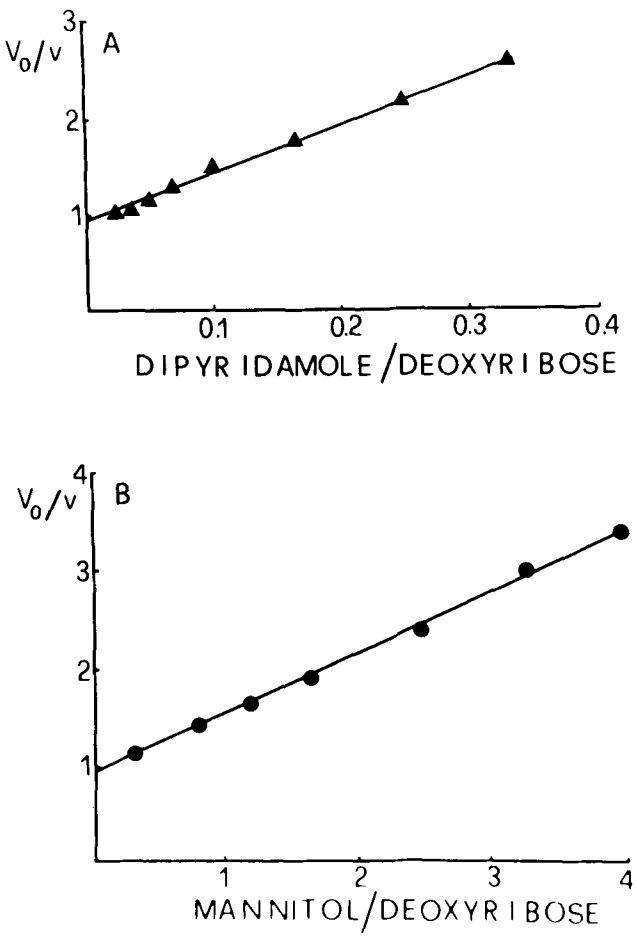

FIG. 6. Competition plots of dipyridamole (plot A) or mannitol (plot B), and deoxyribose with X-rays as radical source of OH•. Concentration of deoxyribose was 2.8 mM. X-radiolysis: 11.3 Gy/min, at 22°C in air atmosphere; pH 5 for dipyridamole, pH 7 for mannitol. Each point represents the mean of three separate experiments carried out in triplicate.

TABLE 1

Second Order Rate Constants for the Reaction of OH• with Dipyridamole and Mannitol

	Rate constant
Dipyridamole[a]	$1.39\text{-}1.93 \times 10^{10}$
Mannitol[a]	$1.45\text{-}2.02 \times 10^{9}$
Mannitol[b]	$1.0\text{-}2.0 \times 10^{9}$
Mannitol[c]	$1.0\text{-}1.8 \times 10^{9}$

[a]This study.

[b]Reference 8.

[c]Reference 11.

hydroxyl radicals (7). Here we report further evidence on the OH•-scavenging activity of dipyridamole. The drug is able to prevent the hydroxylation reaction of salicylate. The formation of both 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate, monitored by HPLC, dependent on hydroxyl radicals generated during water radiolysis, is inhibited by dipyridamole. The reaction of dipyridamole with the hydroxyl radical also is shown by the spectral changes, the more evident of which is the gradual disappearance of the absorption maximum at 404 nm. The rate of reaction of dipyridamole with the hydroxyl radical is considerable ($>10^{10}$ M^{-1} s^{-1}), as determined by the "deoxyribose assay" (8), and is of possible biological interest. Competition kinetics with deoxyribose were carried out both in a Fenton system and with X-radiolysis in order to ensure the validity of the "deoxyribose assay" under our experimental conditions. Second-order rate constants for the reaction of dipyridamole with OH• of 1.72×10^{10} M^{-1} s^{-1} and of 1.54×10^{10} M^{-1} s^{-1} were measured by Fenton chemistry and by radiation chemistry, respectively. Considering the very different nature of the two models, the agreement between the calculated constants is excellent.

Additional support was given by the use of a second internal standard, mannitol, of which the measured rate constants in both the Fenton and radiation chemistry system corresponded to those reported in the literature. The high rate constant (Table 1) for the reaction of dipyridamole with OH• might be attributed to the presence of numerous hydrogen atoms which are abstractable by OH•. In this connection it is interesting that dipyridamole can be considered a carrier of four alcohol groups and so has a resemblance with other OH•-containing scavengers such as mannitol and deoxyribose. An alternative explanation is the occurrence of an addition reaction occurring at the bi-pyrimidine ring with subsequent rupture or saturation. The addition reaction is consistent with the loss of the 404 nm band upon irradiation, however, further studies are needed to elucidate the exact mechanism of the reaction.

The mechanisms of dipyridamole are not yet fully understood even though the drug is already in use in clinical practice as an antiplatelet drug (13). The antioxidant activity of dipyridamole and the powerful scavenging of OH• could be linked to its antithrombotic and vasodilating activity since OFR have been reported to have a role in coronary heart disease (14,15), in platelet activation (16,17) and in the regulation of vascular tone (18,19). The antioxidant activity of dipyridamole could be further utilized therapeutically in other cases where an OFR-mediated mechanism of pathology is involved. Another use could be as *in vivo* OH• probe for conditions such as reperfusion injury where the involvement of OFR has been recognized by measuring OFR by the electron spin resonance spin-trapping technique (14,15).

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Fluorospectroscopic Analysis of the Fluorescent Substances in Peroxidized Microsomes of Rat Liver

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The fluorescent substances formed in rat liver microsomes in the course of lipid peroxidation were investigated by fluorescence techniques. The fluorescence emitted from peroxidizing microsomes continuously increased as lipid peroxidation progressed, while the steady-state fluorescence anisotropy increased and then reached a plateau. A similar increase was observed in the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene in peroxidizing microsomes. The fluorescence from peroxidized microsomes consisted of at least three species having short, middle or long fluorescence lifetimes. The lifetimes and relative amplitudes of fluorescence were unaffected by the extent of lipid peroxidation. Both fluorescence of the chromolipids extracted and the proteins isolated from peroxidized microsomes had the same characteristics in fluorescence lifetimes as the fluorescence from whole peroxidized microsomes. Thus, these lipids and proteins appear to be the major biological substances responsible for the fluorescence emanating from whole peroxidized microsomes. Furthermore, fluorescent substances formed in microsomes seem to increase in quantity rather than change in quality as lipid peroxidation progresses. *Lipids 27,* **354–359 (1992).**

Lipid peroxidation has gained considerable attention due to its serious effects on biological systems. Lipid peroxidation produces various products. Fluorescent substances are one type of characteristic products formed upon lipid peroxidation and they can be observed in artificial phospholipid membranes (1) as well as in biological membranes, such as liver microsomes (2), hepatocytes (3) and red blood cells (4). Tappel (5) and Fletcher *et al.* (6) suggested that lipofuscin, an age pigment, accumulates in tissues in the course of lipid peroxidation in biological membranes. The fluorescence of lipofuscin has been ascribed to reaction products of malondialdehyde (MDA) with amino compounds (5,6). However, some other reactions also have been proposed to form fluorescent substances (7,8). Thiobarbituric acid-reactive substances (TBARS) produced in the membranes during lipid peroxidation, which are commonly used for measuring lipid peroxidation, are mostly released into the medium, with little remaining in the membranes (9–11). However, the fluorescent substances are retained in the membranes and little of it is released into the aqueous phase. The fluorescence emitted from peroxidizing membranes continuously increases as lipid peroxidation progresses, whereas the generation of TBARS ceases at an early stage of lipid peroxidation (11). Therefore, fluorescent substances can be quite useful for evaluating *in vitro* and *in vivo* lipid peroxidation.

Fluorescent substances are commonly characterized by their fluorescence patterns. Shimasaki *et al.* (12) demonstrated that the fluorescence patterns of fluorescent products formed in rat liver phosphatidylcholine liposomes containing glycine during lipid peroxidation were similar to those of conjugated Schiff bases (13) and of the age-related fluorescent substances (14). Fluorescence polarization and lifetime measurements on fluorescent substances were made by Malshet *et al.* (15), and such measurements were found useful for characterizing these substances.

Malshet *et al.* (15) did their measurements on the chloroform/methanol-extractable fluorescent substances. However, the fluorescent substances formed in peroxidizing microsomes not only consist of fluorescent chromolipids but also of fluorescent proteins (11,16). We have previously detected the fluorescence emitted from peroxidized microsomes (11) by a technique which simultaneously gives information on both fluorescent chromolipids and proteins in peroxidized membranes. Most recently, we also have succeeded in determining the lifetimes of fluorescence emitted from peroxidized microsomes without prior extraction (17).

The present study was undertaken to further clarify the properties of fluorescent substances and to analyze the formation of fluorescent substances in peroxidizing microsomes by fluorescence polarization and lifetime measurements.

MATERIALS AND METHODS

Materials. L-Ascorbic acid, $FeSO_4 \cdot 7H_2O$, ethylenediaminetetraacetic acid (EDTA) sodium salt, dithiothreitol and glycerol were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH) and Sephacryl S-400 (superfine) were obtained from Sigma Chemical Co. (St. Louis, MO) and Pharmacia Fine Chemicals (Uppsala, Sweden), respectively. All other reagents were of analytical grade.

Microsomes. Rat liver microsomes were isolated from 170- to 250-g male Wistar rats treated with phenobarbital as previously described (11). The microsomes obtained were suspended in 0.1 M sodium phosphate buffer, pH 7.4. The protein concentration of microsomes was determined by the method of Lowry *et al.* (18) using bovine serum albumin as the standard.

Lipid peroxidation. The microsomes (2 mg protein/mL) were incubated with 0.2 mM ascorbic acid and 10 μM $FeSO_4$ at 37°C. Peroxidation was terminated by adding 1 mM EDTA to the reaction mixture at designated times. The extent to which lipid peroxidation proceeded was determined by the formation of TBARS according to the method of Buege and Aust (19) and expressed as nmoles of MDA equivalents/mg protein. Two milliliters of thiobarbituric acid stock reagent was added to 1.0 mL of the microsome suspension (0.5 mg protein/mL), and boiled for 15 min. The mixture was then cooled and centrifuged at 1,000 × *g* for 10 min, and the precipitate was removed. The absorbance of the samples was determined at 535 nm with a standard curve using tetraethoxypropane.

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; EDTA, ethylenediaminetetraacetic acid; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances.

Extraction of membrane lipids from peroxidized microsomes. Membrane lipids were extracted from the microsomes according to the procedures of Koster and Slee (2). The samples were extracted with chloroform/methanol (1:2, v/v). After centrifugation, chloroform/water (1:1, v/v) was added to the supernatant. The chloroform layer was separated and evaporated under vacuum. Then liposomes were prepared from the extracted lipids according to Bangham *et al.* (20) using a slightly modified method.

Separation of microsomal membrane proteins. Microsomal proteins were separated as previously described (16). The microsomes exposed to the peroxidation reaction *in vitro* for 0, 1 and 5 hr were solubilized according to Imai *et al.* (21). Glycerol (20%), 2% cholic acid and 1 mM dithiothreitol were added to the peroxidized microsomes to solubilize the membranes. The reaction mixture was stirred for 1 hr under cooling with ice and then centrifuged for 1 hr at 105,000 × *g*. Ten mL of the supernatant was applied to a Sephacryl S-400 (superfine) column (Excel column SD 1000) that had been equilibrated with 0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol and 0.5% cholic acid. The rate of elution with the same buffer was 20 mL/hr. Each five mL of the effluent was fractionated. The proteins in the effluent were determined by their absorbance at 280 nm, using a Pharmacia Single Path Monitor UV-1 and by the fluorescence at the excitation wavelength, 355 nm, and emission wavelength, 430 nm, using a Hitachi fluorescence spectrophotometer 650-60 (Tokyo, Japan).

Fluorescence measurements. Control and peroxidized microsomes (0.2 mg protein/mL) in 0.1 M sodium phosphate buffer, pH 7.4, containing 50% glycerol and 1 mM EDTA were measured for fluorescence at 37°C, using the Hitachi fluorescence spectrophotometer 650-60. A cut-off filter (390 nm) was placed on the emission side for each measurement.

Labeling of microsomes with DPH. The microsomes were labeled with DPH as previously described (22). One-half mL of the microsomes (2 mg protein/mL) was incubated for 20 min at 37°C with 4.5 mL of 10 μM DPH in 0.1 M sodium phosphate buffer containing 1 mM EDTA.

Fluorescence anisotropy measurements. Microsomes exposed to the peroxidation reaction *in vitro* for designated reaction times (0–14 hr) were used to measure fluorescence anisotropy. Fluorescent substances and DPH were used as intrinsic and extrinsic fluorophores, respectively. The steady-state fluorescence anisotropy for microsomes in 0.1 M sodium phosphate buffer, pH 7.4, containing 50% glycerol and 1 mM EDTA, was measured at 37°C with the Hitachi fluorescence spectrophotometer 650-60. The samples (0.2 mg protein/mL) were excited by vertically polarized light at 355 nm for fluorescent substances and at 360 nm for DPH. The vertically and horizontally polarized components of fluorescence, which were designated as I_V and I_H, respectively, were measured at 430 nm and 450 nm through the cut-off filter (390 nm) for fluorescent substances and DPH, respectively. Polarizers were used to select the appropriate polarized components for the excitation and emission. The steady-state anisotropy, r^s, was obtained by:

$$r^s = \frac{I_V - GI_H}{I_V + 2GI_H} \quad [1]$$

where G is the ratio of the sensitivity of the detection system for vertically and horizontally polarized light, and is equal to the ratio I_V/I_H obtained by exciting with horizontally polarized light.

Effects of temperature on fluorescence anisotropy. Microsomes were exposed to lipid peroxidation for 0, 1, 5 and 14 hr. The steady-state fluorescence anisotropy of DPH in peroxidizing microsomes was measured by changing the temperature between 5 and 45°C.

Fluorescence lifetime measurements. Fluorescence lifetimes were measured at room temperature using a Horiba fluorescence lifetime instrument (NAES-550) (Kyoto, Japan) which measured lifetime by time-correlated photon counting using a hydrogen flash lamp (17). The samples were excited at 355 nm using a filter of $CuSO_4$ solution (250 g/L) on the excitation side, and the emission light was selected using a cut-off filter (420 nm). These measurements were carried out for the following three samples: i) peroxidized microsomes in 0.1 M sodium phosphate buffer, pH 7.4, containing 50% glycerol and 1 mM EDTA; ii) liposomes made from the extracted lipids in the same buffer as above; and iii) fluorescent protein fractions in 0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol and 0.5% cholic acid. The fluorescence decay curve can be described by the exponential expression:

$$F(t) = \Sigma A_i \exp(-t/\tau_i) \quad [2]$$

where F(t) is the fluorescence at time t, A_i is a relative amplitude of component i which represents the fractional contribution to the fluorescence decay curve and τ_i is the fluorescence lifetime of component i.

Time-resolved decay of the fluorescence anisotropy. The time-resolved decays of anisotropy for the fluorescence emitted from the peroxidized microsomes were measured at room temperature using the fluorescence lifetime instrument. The samples were excited at 355 nm and the emission light was selected using a cut-off filter (420 nm). Polarizers were used to select the appropriate polarized components for excitation and emission. The time-resolved decays of vertically polarized [$I_V(t)$] and horizontally polarized [$I_H(t)$] components of the emission were measured when excited by vertically polarized light. The time-resolved anisotropy [r(t)] is calculated by:

$$r(t) = \frac{I_V(t) - GI_H(t)}{I_V(t) + 2GI_H(t)} \quad [3]$$

Assuming that the rotations of the molecules are symmetric (isotropic) and unhindered, the rotational correlatoin time of the fluorophore, ϕ, is obtained by:

$$r(t) = r_o \exp(-t/\phi) \quad [4]$$

where r_o is the anisotropy which would be observed in the absence of rotational diffusion.

Ultraviolet light irradiation. The samples in the cuvettes for the fluorescence measurements were irradiated with ultraviolet light, using a sterilizing lamp (Toshiba GL 10; wavelength, 253.7 nm; output, 1.8 W) prior to all fluorescence measurements, as previously described (11). The samples in the cuvettes were irradiated at a close distance so that the cuvettes and the lamp nearly touched. All

fluorescence measurements were carried out on 0.2 mg protein/mL of microsomes.

RESULTS

Steady-state fluorescence anisotropy. The fluorescence emitted from microsomes undergoing lipid peroxidation continuously increased over the entire period of the peroxidation reaction (Fig. 1A). The EDTA used to terminate the peroxidation reaction did not affect the fluorescence (data not shown). The generation of TBARS would have been completed well before the end of this long-term experiment (9,11). The steady-state fluorescence anisotropy of the fluorescent substances in the peroxidized microsomes increased with lipid peroxidation and then reached a plateau (Fig. 1B). The fluorescence anisotropy of DPH increased in the same manner as that of the fluorescent substances as lipid peroxidation progressed, as has been shown previously (22).

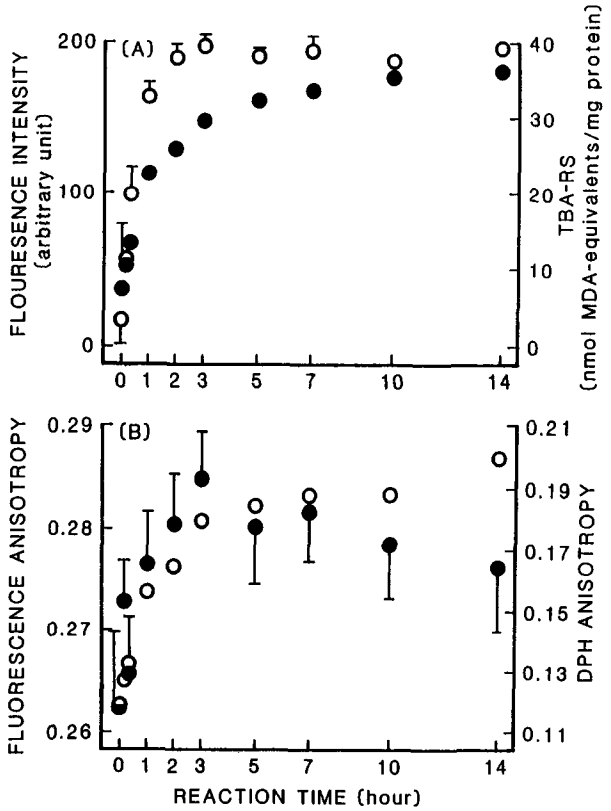

FIG. 1. Time course of chemical and physical changes occurring in microsomes during lipid peroxidation. Lipid peroxidation was induced at 37°C by the addition of 0.2 mM ascorbic acid and 10 μM $FeSO_4$ to the microsome suspension (2 mg protein/mL) and terminated by adding 1 mM EDTA to the reaction mixture at designated times. The following was plotted against lipid peroxidation reaction time: A, formation of fluorescent substances (●) and TBARS (○). B, steady-state fluorescence anisotropy of the fluorescence emitted from microsomes (●) and from DPH in microsomes (○). The data points of TBARS represent the mean values ± SEM of three determinations expressed as MDA-equivalents per mg protein of microsomes. The data points of fluorescence anisotropy represent the mean values ± SEM of six determinations. Where absent, SEM was smaller than the symbols.

Effect of temperature on DPH fluorescence anisotropy. The effect of temperature on the steady-state fluorescence anisotropy of DPH in peroxidized microsomes was examined by changing the temperature between 5 and 45°C (Fig. 2). Eichenberger *et al.* (23) reported a continuous decrease in the DPH anisotropy between 5–37°C for both control and peroxidized rat liver microsomes, suggesting that both types of microsomes were in the liquid-crystalline state over the entire temperature range. In the present study, the control microsomes showed a continuous decrease between 5–45°C. However, the microsomes exposed to lipid peroxidation did not show such a linear decrease. The microsomes exposed to lipid peroxidation for a longer period of time (5 and 14 hr) especially showed a biphasic anisotropy decrease, that is, two lines with different slopes below and above 25–30°C.

Fluorescence lifetime. Fluorescence lifetimes of the fluorescent substances formed in peroxidized microsomes were measured for further characterization. A time-resolved decay of the fluorescence emitted from the microsomes undergoing lipid peroxidation for 5 hr is shown in Figure 3. The data were automatically analyzed according to equation [2] using the built-in program of the fluorescence lifetime instrument. The data of fluorescence decay were fitted best to the fluorescence decay of a three-exponential equation, which was estimated by Chi square (χ^2). The fluorescence lifetimes (τ_i) and relative amplitudes (A_i) of three components are given in Table 1. All decays of the fluorescence from the microsomes undergoing lipid peroxidation for other reaction times also were fitted best to a three-exponential equation. The value of each parameter was plotted against peroxidation reaction time (Fig. 4). The results indicated that at least three species of fluorophore with different fluorescence lifetimes were present. Furthermore, fluorescence lifetime and relative amplitude of each component remained unchanged during the lipid peroxidation process.

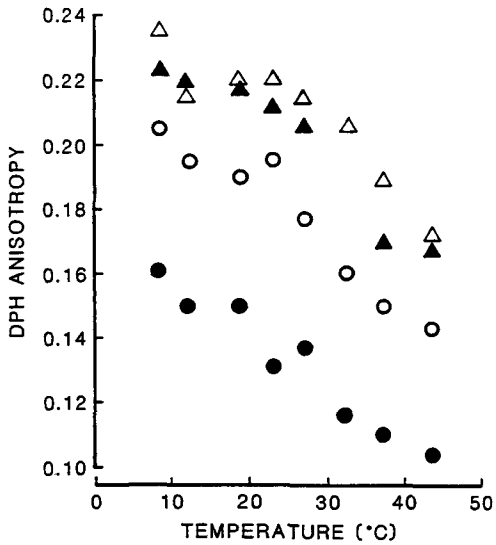

FIG. 2. Temperature dependence of steady-state fluorescence anisotropy of DPH in microsomes. Lipid peroxidation was induced under the conditions given for Figure 1. The microsomes were exposed to lipid peroxidation for 0 hr (●), 1 hr (○), 5 hr (▲)) and 14 hr (△).

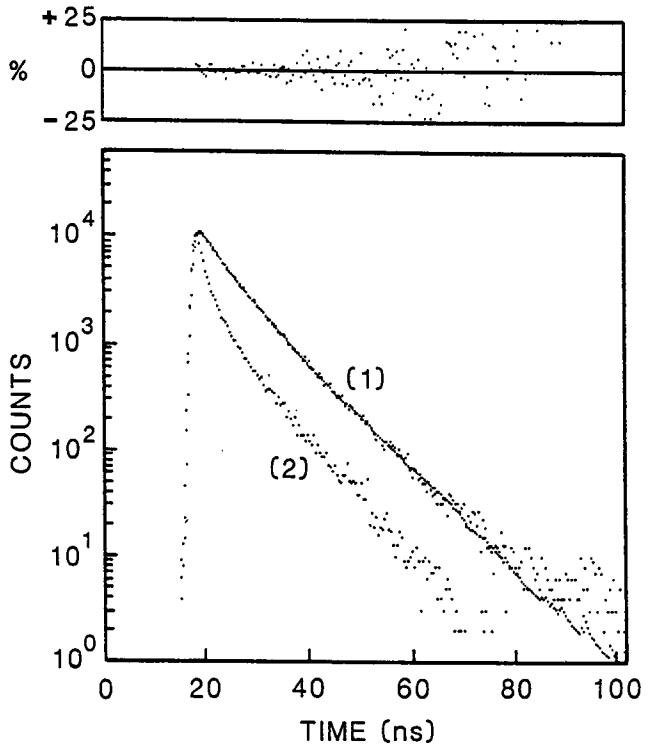

FIG. 3. Fluorescence decay of the fluorescence emitted from peroxidized microsomes. Measurement and analysis of the time-resolved decay of microsomes exposed to lipid peroxidation for 5 hr under conditions given for Figure 1 were carried out as described in Materials and Methods. The decay was fitted best by three components of fluorescence lifetimes. 1, Fluorescence decay (dots: observed values), fitted curve (——); and 2, lamp profile. The residuals of the experimental decay from the fitted curve are shown in the upper trace (%). Chi square (χ^2) in the decay curve was 1.59.

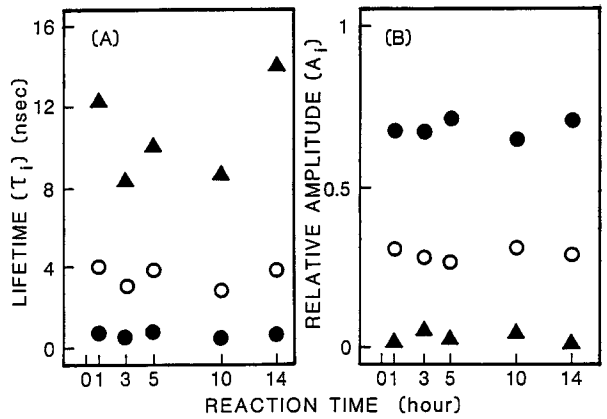

FIG. 4. Fluorescence lifetimes and relative amplitudes of the fluorescence from peroxidized microsomes. Measurement and analysis of the time-resolved decays of the fluorescence from microsomes exposed to lipid peroxidation for various reaction times under the conditions given for Figure 1 were carried out as described in Materials and Methods. All decays gave three components of fluorescence lifetimes. Fluorescence lifetimes and relative amplitudes were plotted against lipid peroxidation reaction time in panels A and B, respectively. Component of fluorescence lifetime: Short fluorescence lifetime (●); middle fluorescence lifetime (○); long fluorescence lifetime (▲).

TABLE 1

Fluorescence Lifetimes and Relative Amplitudes of Peroxidized Microsomes, Lipids and Proteins[a]

Component i	1		2		3	
	τ_1	(A_1)	τ_2	(A_2)	τ_3	(A_3)
Microsomes	0.8	(0.71)	3.9	(0.26)	10.1	(0.03)
Fluorescent chromolipids	0.9	(0.77)	3.7	(0.20)	11.5	(0.03)
Fluorescent proteins	1.0	(0.64)	4.2	(0.32)	12.5	(0.04)

[a]The microsomes underwent lipid peroxidation for 5 hr. The fluorescent chromolipids were extracted from the treated microsomes, and the fluorescent proteins were solubilized and isolated from these microsomes. τ_i, Fluorescent lifetimes; A_i, relative amplitudes.

Fluorescence lifetime of fluorescent lipids. One type of components of fluorescent substances formed in peroxidized microsomes is known to be chromolipids which are extractable with organic solvents (2-4,11). In order to characterize the fluorescent chromolipids by measuring fluorescence lifetimes, liposomes were made from the extracts of microsomes exposed to lipid peroxidation for 5 hr. Fluorescence decays of the extracted chromolipids were analyzed according to equation [2] and were found to fit best with a three-exponential equation. The fluorescence lifetime and relative amplitude for each component are given in Table 1. The extracted chromolipids from the microsomes exposed to lipid peroxidation for 1 and 10 hr gave similar results in regard to fluorescence lifetime and relative amplitude of each of the components.

Fluorescence lifetime of the fluorescent proteins. The microsomal proteins are another important type of component which can contribute to the fluorescence emitted from peroxidized microsomes (16). The microsomes undergoing lipid peroxidation were solubilized with cholic acid and fractionated by the Sephacryl S-400 gel column chromatography. The elution pattern of proteins solubilized from the microsomes after peroxidation for 5 hr is shown in Figure 5. The F-fraction containing the fluorescent proteins was used for the fluorescence lifetime measurements; the fluorescence of this F-fraction increased as lipid peroxidation progressed (16). The fluorescence decays of the fluorescent proteins (F-fraction) were analyzed according to equation [2] and fitted best with a three-exponential equation. The parameters obtained by this analysis are given in Table 1. Similar values were obtained for the microsomes exposed to lipid peroxidation for 1 hr. Thus, the values were quite independent of lipid peroxidation time.

Time-resolved decay of the fluorescence anisotropy. Finally, the time-resolved decays of anisotropy for the fluorescence from peroxidized microsomes were examined (Fig. 6). The decays are defined as given in equation [3]. The fluorescence decays of anisotropy were analyzed only for the short component of the fluorescence lifetime, using equation [4]. The calculated rotational correlation times of the fluorophore in microsomes exposed to lipid peroxidation for 1, 5 and 10 hr were 45, 71 and 93 ns, respectively.

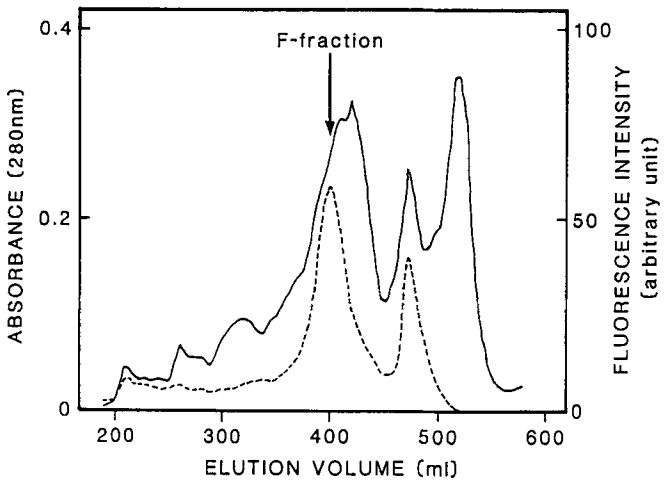

FIG. 5. Fractionation of fluorescent proteins of peroxidized microsomes. The proteins solubilized from microsomes exposed to lipid peroxidation for 5 hr were fractionated on a Sephacryl S-400 gel column. Each fraction of the effluent (5 mL) was determined by the absorbance at 280 nm (——) and the fluorescence (excitation wavelength, 345 nm; emission wavelength, 430 nm) (- - -). The fraction containing fluorescent proteins indicated by an arrow was designated F-fraction.

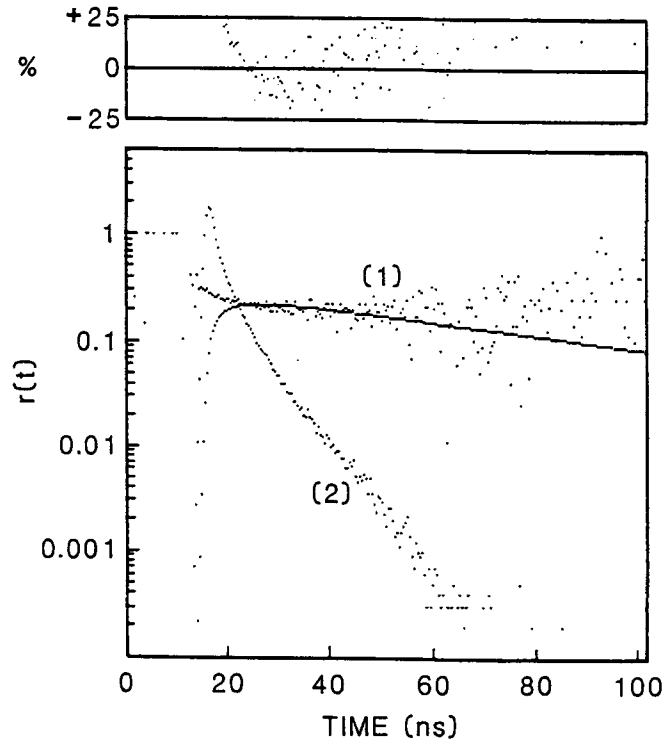

FIG. 6. Time-resolved fluorescence anisotropy decay of the fluorescence from peroxidized microsomes. Measurement and analysis of the time-resolved fluorescence anisotropy of the fluorescence emitted from microsomes exposed to lipid peroxidation for 5 hr were carried out as described in Materials and Methods. 1, Fluorescence anisotropy (dots: observed values), fitted curve (——); and 2, lamp profile. The time-resolved anisotropy was analyzed only for the short component, because the photon counts were insufficient for analyzing other components accurately. The residuals of the experimental decay curve from the fitted curve are shown in the upper trace (%).

DISCUSSION

Lipid peroxidation in biological membranes is accompanied by the formation of fluorescent substances and is suggested to induce the accumulation of lipofuscin in tissues (5,6,24). Fluorescence measurements *in situ* directly on peroxidized microsomes is a very useful approach to studying the changes that occur in membranes upon lipid peroxidation (11). Peroxidized lipids and proteins are the two major components giving rise to fluorescence in peroxidized microsoms (11,16), and the present technique can simultaneously provide information on both types of fluorescent materials without prior separation.

The formation of fluorescent substances showed a pattern which was quite different from that of TBARS formation. We found that the fluorescence emitted from peroxidized microsomes increased continuously during the entire process of lipid peroxidation (Fig. 1A) (11). This increase may be due to an increase of amount of fluorescent substances present and/or to an increase in the fluorescence quantum yield of fluorescent substances produced.

The time-resolved fluorescence decays were measured to characterize the fluorescent substances. The fluorescence from peroxidized microsomes consisted of at least three components of about 1, 4 and 10 ns lifetimes. The peroxidation time did not alter these lifetimes and the relative amplitudes (Fig. 4). Thus, the continuing increase in fluorescence from peroxidized microsomes thus appears to be not due to a change in the quality of the fluorescent substances, but due to an increase in quantity.

The major chemical components of fluorescent substances formed in peroxidized microsomes are the fluorescent chromolipids (2,11) and proteins (16). The time-resolved fluorescence decays of these two major types of membrane components were examined to reveal how these components contribute to the fluorescence from whole peroxidized microsomes. Both fluorescent chromolipids extracted and fluorescent proteins isolated from peroxidized microsomes had three species of fluorescence lifetime. Each lifetime value and the respective relative amplitude obtained from the fluorescent chromolipids and proteins were similar to those of the fluorescence emitted from whole peroxidized microsomes. Interestingly, the values for the fluorescence lifetimes and relative amplitudes of the fluorescent chromolipids and proteins were not affected by the peroxidation time, which was the same as in whole peroxidized microsomes. This confirms that the fluorescent substances increase in their amount rather than change in their quality as lipid peroxidation progresses. Furthermore, each of three different lifetime components increases with progressing lipid peroxidation, keeping their relative ratio constant throughout.

The steady-state fluorescence anisotropy for the fluorescence from whole peroxidized microsomes has now been measured for the first time (Fig. 1B). The anisotropy showed a saturable increase with increasing lipid peroxidation. This increase can possibly be attributed to a change in the fluorescent substances themselves and/or in the microenvironment surrounding the fluorescent substances. The fluorescence anisotropy of DPH indicated decreasing fluidity of the microsomal membranes upon lipid peroxidation (22,23,25). This change in fluidity possibly may contribute to the increased anisotropy of the fluorescence from peroxidized microsomes, because both

fluorescent substances and DPH showed a similar profile for the anisotropy *vs.* peroxidation time (Fig. 1B). This increase of the fluorescence anisotropy for peroxidized microsomes evidently is not due to a change in the fluorescence lifetimes, because the lifetimes were unchanged during lipid peroxidation. Furthermore, the rotational correlation times increased with lipid peroxidation. These results indicate that the rotational motion of the fluorophore in peroxidized microsomes is depressed upon lipid peroxidation. The decreased fluidity of microsomal membranes appears to affect the rotational motion of the fluorophores in microsomes. Moreover, a change in the fluorescent substances themselves, *e.g.*, the molecular size and/or shape, would affect the rotational motions of the fluorophores.

The occurrence of lipid peroxidation in microsomes inactivates membrane-bound enzymes such as glucose-6-phosphatase and UDP glucuronyltransferase (22,26). The microsomal monooxygenase system also is affected by lipid peroxidation (25,27,28). Cytochrome P-450 may be involved in fluorscent proteins formed in peroxidized microsomes (16). Thus, formation of fluorescent substances may affect various physiological functions of microsomes not only through a change in physical properties of the microsomal membranes, but also through a transformation of enzymes into fluorescent substances.

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A Study of the Composition of Fish Liver and Body Oil Triglycerides

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Silver-ion high-performance liquid chromatography (Ag^+-HPLC) was used to study the range and variations in molecular species of triglycerides from industrial, retail and laboratory extracted fish oils. These were contrasted with a typical plant oil. Selected fish oils were fractionated and the fatty acid distribution of the fractions determined by gas-liquid chromatography. Fish oils gave a characteristic Ag^+-HPLC profile, typified by sharp, intense peaks at the start of the chromatogram and broad, multiple nongaussian peaks for the late eluting components. Triglycerides ranging from those that were wholly saturated to those containing 16 double bonds were isolated. Cod (*Gadus* spp.), saithe (*Pollachius virens*) and monkfish (*Squatina squatina*) liver oils gave similar triglyceride profiles. Mackerel (*Scomber scombrus*), capelin (*Mallotus villosus*) and herring (*Clupea harengus*) body oils gave characteristic triglyceride profiles which were associated with high concentrations of 20:1 and 22:1 fatty acids. Only small amounts of these particular triglycerides were observed for menhaden (*Brevoortia* spp.), South African anchovy (*Engraulis capensis*) and Indian sardine (*Sardinella longiceps*) oils, all of which contained minor amounts of these acids. The latter oils contained highly unsaturated triglycerides, whereas only traces of these were noted for the former. Chromatography with Ag^+-HPLC can be used for the rapid screening of fish oils and for selecting those oils rich in polyunsaturated acids that may be suitable for enrichment. Cottonseed oil gave well-defined and discrete peaks. Similar peaks were observed in the chromatogram of Omega-combination, a mixture of primrose and fish oils. Thus, fish, plant and a mixture of these oils can be readily distinguished.
Lipids 27, 360–370 (1992).

An intense interest in the health benefits of fish oils was stimulated in the early 1970s by the research of Dyerberg and co-workers (1–4). The n-3 polyunsaturated fatty acids (PUFA) *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA, 20:5) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA, 22:6) are the active components of such oils [for reviews of n-3 biochemistry and clinical applications, see (5,6)]. A substantial number of fish oil nutritional supplements are available (7), many of which are defined as "concentrates." There is considerable interest in producing fish oils enriched with PUFA for use as pharmacological agents, but a better understanding of the triglyceride composition of crude and refined fish oils is required.

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Abbreviations: Ag^+-HPLC, silver-ion high-performance liquid chromatography; BHT, butylated hydroxytoluene; BP, British Pharmacopoeia; CLO, cod liver oil; DHA, *cis*-4,7,10,13,16,19-docosahexaenoic acid; EPA, *cis*-5,8,11,14,17-eicosapentaenoic acid; GLC, gas-liquid chromatography; GLC/MS, gas-liquid chromatography/mass spectroscopy; HPLC, high-performance liquid chromatography; PUFA, polyunsaturated fatty acid(s); TLC, thin-layer chromatography; TMAH, tetramethylammonium hydroxide; UCM, unresolved complex mixture.

High-performance liquid chromatography (HPLC), both normal and reversed-phase, has been increasingly used to study lipids and more specifically triglycerides (8–12). Silver ions have been added to the solvent (13) to modify selectivity. Detection has always been a problem in HPLC, but the advent of the mass (light-scattering) detector has greatly facilitated the use of HPLC for lipid analysis (14–18). Christie (19,20) has developed a method for the analysis of triglycerides using silver-ion high-performance liquid chromatography (Ag^+-HPLC) and mass detection. This technique permits rapid and reproducible analyses of triglycerides which can be separated on the basis of the number of double bonds per triglyceride. The large range of fatty acids present in fish oils results in a complex mixture of molecular species of triglycerides, but Laakso *et al.* (21) have utilized a silver ion column to study the triglycerides in oils from Atlantic herring (*Clupea harengus*), the closely related Baltic herring, and sand eel (*Ammodytes* spp.). A more detailed study of fish oil triglycerides requires more than one chromatographic technique, and Laakso and Christie (22) have combined silver-ion and reverse-phase HPLC to study the triglycerides of Atlantic herring.

In the work described here, a range of industrial, retail and laboratory-extracted fish oils were analyzed by gas-liquid chromatography (GLC) and Ag^+-HPLC with the aim of studying the range and variations in molecular species of triglyceride present in such oils. More detailed investigations of the triglyceride composition of three of these fish oils included GLC analysis of fractions isolated by HPLC. Through an increased understanding of the triglyceride composition of fish oils, the ultimate aim is to develop a rapid and economic method for enriching fish oils and from the HPLC profile predict the suitability of a crude fish oil for enrichment.

MATERIALS AND METHODS

Chemicals. Hexane, methanol and acetonitrile were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Acetone and 1,2-dichloroethane were "Hipersolv" grade from BDH Chemicals Ltd. (Poole, England). Copper(II)sulfate, sodium sulfate and orthophosphoric acid were "AnalaR" grade reagents from BDH Chemicals Ltd. Chloroform and diethyl ether were Pronalys AR reagents from May and Baker (Dagenham, England). Tetramethylammonium hydroxide (TMAH, 20% solution in methanol) was obtained from Aldrich Chemical Company Ltd. (Dorset, England). All lipid standards and boron trifluoride (15% solution in methanol) were purchased from Sigma Chemical Company Ltd. (Poole, England).

Fish oils. Cod (*Gadus* spp.) liver oil BP, saithe (*Pollachius virens*) liver oil, monkfish (*Squatina squatina*) liver oil, a Russian fish oil of unknown provenance, and orange roughy (*Hoplostethus atlanticus*) oil were provided by Isaac Spencer and Co. Fleetwood (1920) Ltd. (Fleetwood, England). Menhaden (*Brevoortia* spp.) oil was obtained from the Zapata Haynie Corporation (Reedville, Virginia). Sun'n'Sea cod liver oil (Cupal Ltd., Blackburn, England) and Seven Seas cod liver oil (Seven Seas Health Care Ltd., Hull, England; batch 81159) were purchased

from a local retailer, as were Marine 25 and Omega-combination oil capsules which had been prepared by Callanish Ltd. (Breasclete, Isle of Lewis, Scotland). Oil from Nile perch (*Lates albertianus*), a Chilean fish oil, South African anchovy (*Engraulis capensis*) oil and Indian sardine (*Sardinella longiceps*) oil were provided by Dr. R. Hardy and Mr. P. Howgate, Torry Research Station (Aberdeen, Scotland).

Cottonseed oil. Cottonseed oil (batch Q80505) was purchased from Larodan AB (Malmo, Sweden).

Extraction of oil from capelin, mackerel, cod livers and herring. Whole capelin (*Mallotus villosus*) were defrosted and minced in a Kenwood Chef mincer. The mince (474.3 g) was mixed with sodium sulfate (142.3 g) and centrifuged at 1150 × *g* for 20 min at 3°C (Fisons MSE Coolspin centrifuge, Crawley, England). The resulting oil was poured off and passed through a layer of sodium sulfate under a nitrogen atmosphere. The yield was 16.9 g (3.6%).

A single mackerel (*Scomber scombrus*) caught off the north of Scotland in October was defrosted under cold running water, and the head and tail were removed. The fish was minced, and the mince (390.9 g) was mixed with sodium sulfate (117 g); the sample was treated as for the capelin. The yield was 24.6 g (6.3%).

Cod (*Gadus morhua*) livers taken from cod landed at Gourden, northeast Scotland in mid-December were thawed, homogenized and weighed. Anhydrous sodium sulfate (30%, w/w) was intimately mixed with the homogenate and the mixture was treated as before. The oil was stored in 15-mL aliquots under a nitrogen atmosphere at −28°C. This sample was coded CLO 3/4.

Fresh herring (*Clupea harengus*), caught off the west coast of Scotland during July, were gutted, filleted and the fillets minced. The fish muscle mince (50 g) was extracted by the method of Bligh and Dyer (23) in the presence of butylated hydroxytoluene (BHT) and the oil stored at −60°C under nitrogen.

Silver-ion high-performance liquid chromatography of fish oils. HPLC analyses were performed with a Vista 5500 liquid chromatograph (Varian UK Ltd., Surrey, England) together with an ACS Model 750/14 mass detector (Applied Chromatography Systems, Macclesfield, England). The Vista 5500 was programmed using a Varian Vista 402 Data Station which also recorded the chromatograms. The HPLC column (25 cm × 0.46 cm) was packed with Nucleosil™ 5SA (HPLC Technology Ltd., Cheshire, England) using a Shandon column packer (Shandon Scientific Ltd., Cheshire, England) and conditioned with silver ions according to the method of Christie (19).

The triglycerides were resolved using a complex ternary solvent system comprised of 1,2-dichloroethane (A), acetone (B), and acetone/acetonitrile (75:25, v/v; C). The specific gradient was dependent on the condition of the column. Typical parameters are given in Table 1. The flow rate of the mobile phase was 1 mL/min.

The fish oils were dissolved in 1,2-dichloroethane and 50 μL (600 μg) were injected onto the column. The retention times of triglycerides with varying degrees of unsaturation were assessed using tripalmitin, 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol, 1,3-distearoyl-2-oleoyl-*rac*-glycerol, 1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol, triolein, trilinolenin and triarachidonin. The standard triglycerides were run on their own and mixed with the Russian fish oil.

The resolution of the column deteriorated progressively over 50 hr of analysis and was influenced by the column standing overnight in 1,2-dichloroethane. Thus, once the gradient had been established, the fish oils were analyzed on a continuous basis using an autosampler. At regular intervals, the column performance was assessed using the Russian fish oil. In addition, blank injections of 1,2-dichloroethane were made to ensure that no carry-over of sample was occurring with the autosampler.

TABLE 1

Typical Gradient Conditions for Elution of Triglycerides from Fish Oils[a]

Time (min)	Solvent		
	% A	% B	% C
0.0	100	0	0
15.0	50	50	0
20.0	20	60	20
30.0	0	75	25
33.0	0	60	40
50.0	0	40	60
55.0	0	0	100
75.0	0	0	100
80.0	100	0	0
100.0	100	0	0

[a]The period from 75.0 to 100.0 min was required to recondition the column. A, 1,2-dichloroethane; B, acetone; C, acetone/acetonitrile (75:25, v/v).

The retention times for wax esters were determined using palmityl palmitate, oleyl palmitate, oleyl oleate and orange roughy oil (95% wax esters). A similar analysis was carried out on a sample of free fatty acids prepared by saponification of cod liver oil (Seven Seas, batch 81159) with ethanolic potassium hydroxide (1 mol/L).

Fractionation of fish oils by HPLC. Fish oils were chromatographed as already described, the detector output being recorded on a Chromjet integrator (Spectra Physics, Ltd., Hemel Hempstead, England). A stream-splitter (Thames Chromatography, Berkshire, England) was then adjusted such that 30% of the column eluant was directed to the detector and a second sample of the same fish oil was chromatographed. Fractions were collected manually, according to the times obtained from the preliminary run but with slight variations being made in accordance with the actual emerging HPLC profile. The fractions were stored in screw-capped tubes at −65°C until they were methylated, using TMAH. The three oils treated in this way were the Russian fish oil, Indian sardine oil and a Seven Seas cod liver oil (batch 811509).

Preparation of methyl esters. Fatty acid methyl esters of the fish oils were prepared using boron trifluoride in methanol as described previously (24). Preparation of methyl esters from the HPLC fractions was performed by base-catalyzed transesterification using TMAH (25). Methyl tricosanoate (100 μL; 60 μg/mL) was added as a standard to assess recoveries prior to the TMAH (0.2 mL) and the reaction mixture was shaken at room temperature using an IKA-VIBRAX-VXR (Janke and Kunkel, Surrey, U.K.) for 4 min. The extracted methyl esters were dissolved in hexane (0.2–0.4 mL) and stored at −60°C until they were analyzed by GLC.

Gas-liquid chromatography and gas-liquid chromatography/mass spectroscopy (GLC/MS). GLC analyses of

fatty acid methyl esters were performed on a Hewlett-Packard 5880A capillary gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) as described previously (24). Individual fatty acids were identified by reference to a standard fish oil (EEC interlaboratory calibration study). GLC/MS analyses of the fatty acid methyl esters were performed using an Autospec high-resolution mass spectrometer (VG Analytical, Manchester, England) as described previously (24).

Thin-layer chromatography (TLC). Fish oils were analyzed by thin-layer chromatography on LHP-K linear high-performance TLC plates (Whatman Labsales Ltd., Kent, England). The samples (4 mg/mL) were applied using Drummond microcaps (1 μL; Drummond Scientific Company, Broomall, PA) and the plate developed with hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The lipids were visualized by spraying with copper(II)sulfate (10%, w/v) in aqueous orthophosphoric acid (80%, v/v) followed by heating at 180°C for 10 min. The samples were run against authentic standards.

RESULTS

Fatty acid compositions of the fish oils and cottonseed oil. The fatty acid compositions of the fish oils and of a cottonseed oil are summarized in Table 2.

TABLE 2

Fatty Acid Composition (area % of total detected area) of the Fish Oils and Cottonseed Oil[a]

	Commercial liver oils					Unrefined liver oil	Natural marine oils		
Fatty acid	Seven Seas cod liver oil	Sun'n'Sea cod liver oil	Isaac Spencer cod liver oil	Saithe liver oil	Monkfish liver oil	3/4 Cod liver oil	Mackerel body oil	Capelin oil	Herring body oil
14:0	6.3	5.5	9.2	4.8	4.1	3.6	8.2	6.8	7.4
15:0	Tr	Tr	0.9	0.4	0.4	0.3	0.6	Tr	Tr
16:0	16.0	10.2	26.2	15.4	13.9	12.1	12.6	10.6	13.8
16:1	8.0	6.7	8.8	6.4	6.3	4.5	3.4	9.5	7.5
16:2	0.6	0.5	0.7	Tr	—	0.3	Tr	0.5	0.8
16:3	Tr	0.7	Tr	Tr	—	Tr	Tr	Tr	0.6
16:4	0.9	1.1	Tr	Tr	Tr	0.3	Tr	0.9	1.1
18:0	2.8	1.4	4.6	3.0	2.5	2.8	1.5	0.8	1.2
18:1	25.0	13.3	14.8	23.4	21.1	17.0	9.6	11.2	9.9
18:2	4.4	0.9	1.7	1.6	1.7	1.4	2.2	1.1	0.9
18:3	1.8	0.4	0.6	1.0	1.2	0.9	2.3	0.5	0.6
18:4	2.2	2.2	2.6	2.3	3.6	2.4	6.1	2.1	2.2
20:0	Tr	—	Tr	—	—	—	—	—	Tr
20:1	5.7	17.5	1.1	10.4	8.7	8.2	10.6	19.1	10.3
20:4	1.3	0.4	Tr	0.7	2.0	1.6	1.9	Tr	0.5
20:5	10.4	12.1	7.0	7.0	7.7	15.6	6.6	7.8	10.1
22:1	3.5	13.4	4.3	10.1	10.0	8.1	15.8	22.7	20.7
21:5	Tr	0.5	—	—	Tr	0.4	Tr	Tr	0.5
22:5	1.8	1.1	0.9	1.0	1.4	1.4	1.1	0.5	0.9
22:6	8.5	8.8	7.5	12.4	14.2	16.6	12.8	3.9	10.0

	Industrially produced marine oils					Special oils			Plant oil
Fatty acid	Russian fish oil	Chilean fish oil	South African anchovy oil	Indian sardine oil	Menhaden fish oil	Nile perch (freshwater)	Marine 25 (concentrate)	Omega combination (mixed fish/vegetable)	Cottonseed
14:0	7.6	6.4	10.5	10.8	7.9	3.4	11.7	5.6	0.7
15:0	Tr	0.8	0.5	1.0	0.6	0.9	Tr	Tr	—
16:0	18.2	20.1	16.0	21.5	19.8	25.6	13.2	9.4	23.8
16:1	6.9	5.8	11.3	10.2	9.8	14.7	11.3	5.4	0.6
16:2	0.6	0.5	1.1	1.3	1.3	—	1.2	0.5	—
16:3	Tr	Tr	1.1	2.2	1.4	—	0.9	0.5	—
16:4	1.5	0.7	2.5	1.2	0.8	—	3.7	1.7	—
18:0	2.1	4.2	2.8	4.2	3.4	7.9	2.4	2.1	2.4
18:1	13.6	17.7	10.1	8.4	12.6	24.7	11.9	13.5	19.5
18:2	1.2	1.4	1.0	1.2	1.3	2.2	0.9	37.3	52.5
18:3	0.7	0.8	0.4	0.5	1.2	1.2	0.3	3.9[b]	0.5
18:4	3.0	2.1	1.5	2.5	3.9	—	1.6	0.8	—
20:0	—	—	0.4	0.7	Tr	Tr	0.7	0.5	Tr
20:1	5.7	1.3	0.5	Tr	1.3	Tr	0.8	0.4	—
20:4	1.7	1.7	1.7	2.5	2.0	1.7	1.3	0.4	—
20:5	17.4	13.4	24.5	18.6	12.7	2.1	28.9	13.7	—
22:1	6.0	Tr	1.0	—	1.4	Tr	0.6	Tr	—
21:5	0.5	Tr	0.8	0.7	0.6	Tr	0.8	0.4	—
22:5	2.2	3.1	1.9	1.6	2.0	3.6	2.6	1.3	—
22:6	10.2	18.4	9.7	8.6	11.2	5.9	5.4	2.6	—

[a]The percentages include the total for various isomers where appropriate (*e.g.* 20:4n-6 and 20:4n-3). Branched and odd-numbered carbon fatty acids, other than 15:0, are not included; hence the totals do not sum to 100%. Tr, trace (<0.3%).

[b]In evening primrose oil, the 18:3 is the *all-cis*-6,9,12-octadecatrienoic acid.

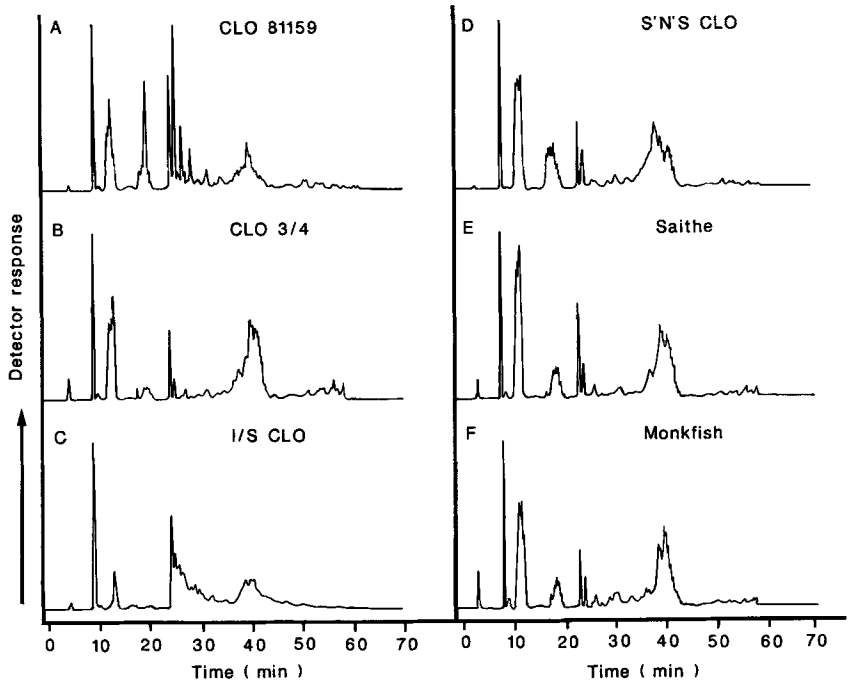

FIG. 1. Silver-ion HPLC of the triglycerides from fish liver oils. A, Seven Seas cod liver oil (batch 81159); B, laboratory-extracted cod liver oil (CLO); C, Isaac Spencer cod liver oil; D, Sun'n'Sea cod liver oil; E, saithe liver oil and F, monkfish liver oil. The oils (120 mg) were dissolved in 1,2-dichloroethane (10 mL). Injections (50 μL, 600 μg) were performed automatically to ensure reproducible reconditioning of the column and enabled all oils to be analyzed on a continuous basis. This ensured that the retention times of specific triglycerides remained constant.

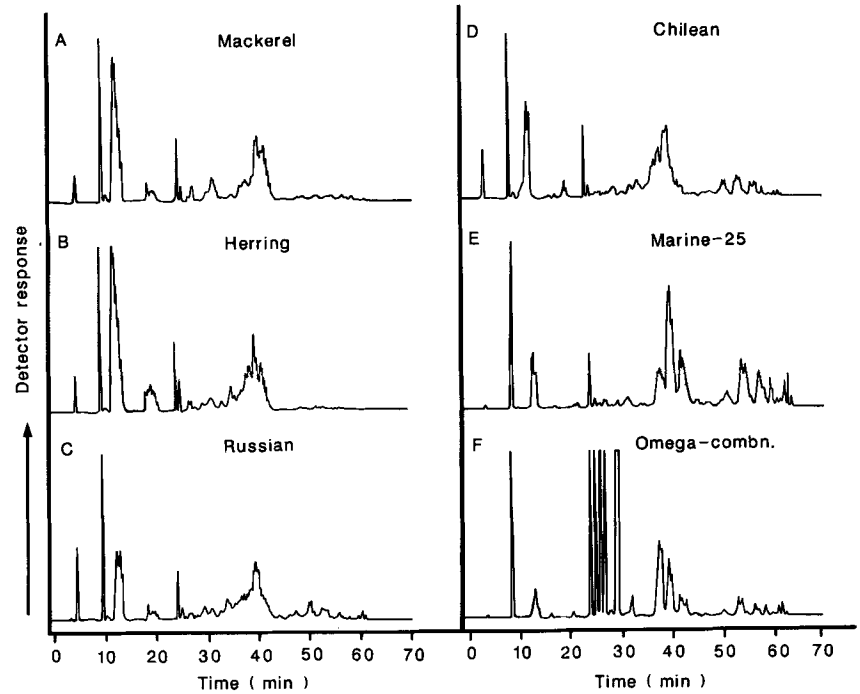

FIG. 2. Silver-ion HPLC of triglycerides from fish body oils. A, mackerel; B, herring; C, Russian fish oil; D. Chilean fish oil; E, Marine-25; F, Omega-combination. The Omega-combination includes both fish and plant oils.

Commercial cod liver oils are refined and clarified by filtration at 0°C (26). The removal of saturated triglycerides produces subtle differences between natural cod liver oil triglycerides and those purchased retail. Variations in the fatty acid composition resulting from season (27), fishing ground and sex (28) mean that direct comparison of the fatty acid composition of various cod liver oils is of little relevance, although typical ranges are recognized for specific fatty acids (27,28). The Issac Spencer cod liver oil, which was produced from cod caught off Iceland, was exceptional in that the level of eicosenoic acid (20:1) was extremely low (1.1%) while that for hexadecanoic acid (16:0) was very high. The presence of two unidentified components, which eluted after 22:6, resulted in the tabulated fatty acids adding up to only 90.8%. Adjustment of the figures by excluding these compounds did not produce a significant increase in the level of 20:1.

The mackerel and capelin body oils, and the herring fillet oil, contained large quantities of 20:1 and docosenoic acid (22:1). This contrasts with the oils produced industrially from the bodies of the fish from other regions, which contained only trace amounts of 20:1 and no 22:1. The Indian sardine oil, the South African anchovy oil, and the menhaden fish oil thus contained very small amounts of these monoenoic acids. These results are similar to those of Ackman (29,30). The lipid from Nile perch, a freshwater fish, was a very soft solid at room temperature, and the combined percentage for 20:5 and 22:6 (8.0%) was considerably less than for the marine fish oils.

Marine 25, a fish oil concentrate, contained the highest percentage of EPA (28.9%) of any of the fish oils, but the proportion of DHA (5.4%) was relatively low. Omega-combination contained a mixture of fish oil and evening primrose oil. The presence of the plant oil was reflected in the amount of *cis,cis*-9,12-octadecadienoic acid (18:2) in this oil. Furthermore, the octadecatrienoic acid is the *cis,cis,cis*-6,9,12-18:3 isomer (31).

TLC analysis of the fish oils. Trace amounts of wax esters, free fatty acids and sterols were detected in the fish oils.

Ag^+-HPLC of the fish oils and cottonseed oil. The liver oils (Fig. 1 A–F), with the exception of the Isaac Spencer cod liver oil (Fig. 1C), gave similar overall profiles. The commercial cod liver oils gave only a small trisaturated triglyceride peak (4 min) compared to the laboratory oil. This would be expected for a cold-cleared oil. The peaks centered at 9 min and 11.5 min were coincident with the standards 1,2-dipalmitoyl-3-oleoyl-*rac*-glycerol and 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol, respectively.

An unresolved complex mixture (UCM), centered around 40 min, was a characteristic feature of the chromatograms. Cod liver oil 3/4, which contained at least 1.5 times as much 20:5 and 22:6 as the other liver oils, had the most significant peaks eluting after 50 min. These peaks were all of low amplitude. This was a result of the low concentration of triglycerides that contain highly unsaturated fatty acids and also of a decrease in detector response. The response of the mass detector is not constant for the different molecular species of triglyceride. The limit of detec-

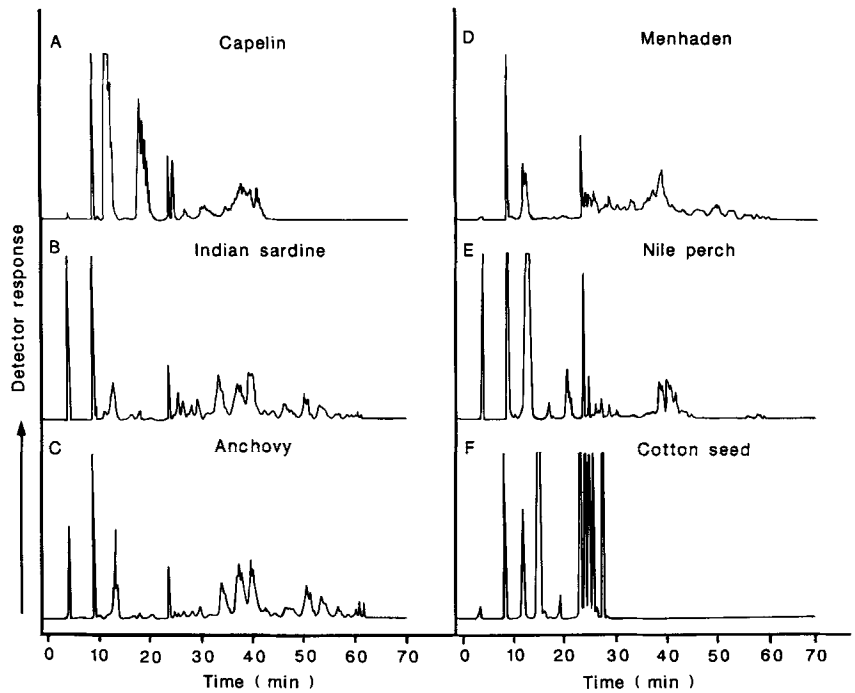

FIG. 3. Silver-ion HPLC of triglyceride from fish oils and cottonseed oil. A, capelin; B, Indian sardine; C, South African anchovy; D, menhaden; E, Nile perch; F, cottonseed.

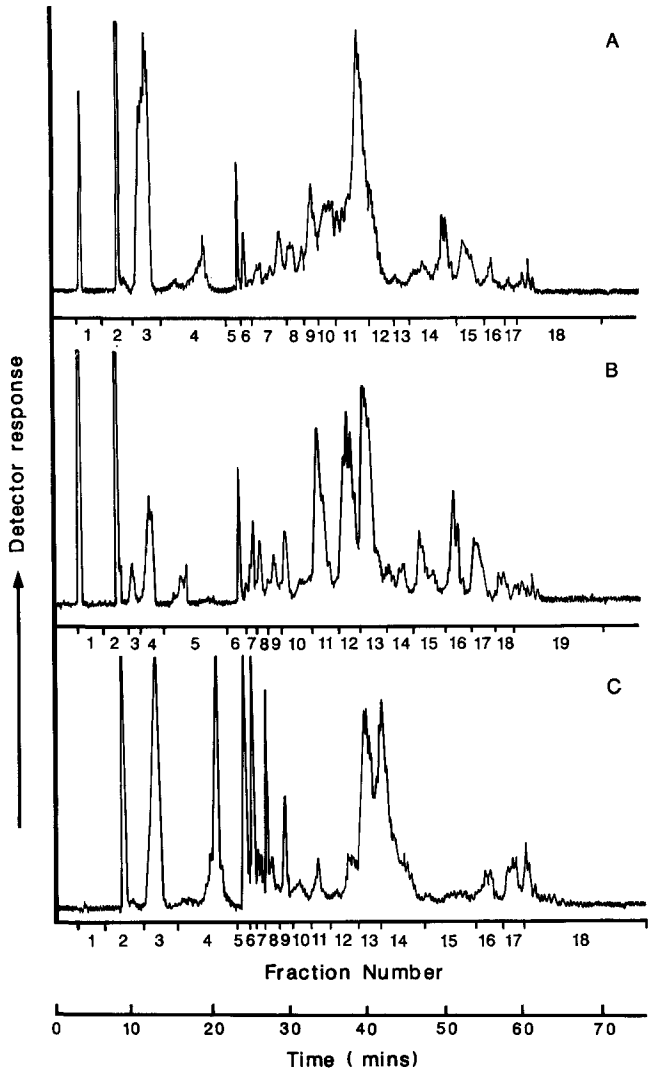

FIG. 4. HPLC of A, Russian fish oil; B, Indian sardine oil and C, Seven Seas cod liver oil (batch 811509) triglycerides. The column eluant passed through a stream-splitter and fractions were collected and numbered as indicated in the diagram. An incrased integrator sensitivity was used to provide greater detail of the triglyceride patterns.

tion was 1.5 μg and 0.8 μg for trilinolenin and tripalmitin, respectively. The equivalent figure for palmityl palmitate was 0.8 μg. In addition, 2.6 μg trilinolenin gave 4267 integrator counts, which was one-tenth of the response from an equivalent amount of tripalmitin (Moffat, Colin, unpublished results). Although a linear response was observed for the wax ester and a given triglyceride between the limit of detection and 25 μg, the response decreased for concentrations in excess of 25 μg. In addition, the gradients of the regression lines for the plots of detector response against amount of triglyceride increased going from trilinolenin to tripalmitin. In the case of trilinolenin, the detector response was proportional to the square root of the concentration over the range 25 to 500 μg.

The mackerel, herring, Russian and Chilean fish oils gave similar overall patterns (Fig. 2A–D). The chromatogram for the herring fish oil was similar to that obtained by Laakso *et al.* (21). Marine 25 (Fig. 2E) contained 32.3% pentaenoic acids and 5.4% hexaenoic acids. This oil gave several large peaks eluting after 50 min.

Christie (19) has shown that vegetable oils such as palm oil give more discrete peaks than fish oils when using a silver-loaded column. Omega-combination contained both fish oil and a primrose oil as clearly illustrated by the HPLC separation (Fig. 2F). Both the fish oil derived triglycerides with their characteristic non-gaussian peak profiles and the discrete peaks typical of a plant oil, as exemplified by the cottonseed oil (Fig. 3F), and observed for corn oil, peanut oil and olive oil (Moffat, Colin, unpublished results) were evident.

Capelin oil (Fig. 3A) contained substantial peaks around 10–13 min and 16–20 min. No triglycerides were eluted after 50 min. This corresponded with the high monoenoic acid (62.5%) and low pentaenoic/hexaenoic acid (12.2%) content of the oil. In contrast, the Indian sardine oil, which contained only 18.6% monoenes, gave small triglyceride peaks between 10 and 13 min with only a trace of triglycerides around 18 min (Fig. 3B). The triglyceride profile for the anchovy oil (Fig. 3C) was similar to the Indian sardine oil while the menhaden oil (Fig. 3D) was more like the Chilean fish oil (Fig. 2D).

Triglycerides containing three saturated acids, two saturated and one monoenoic acid, one saturated and two monoenoic acids dominated the HPLC profile for the Nile perch oil (Fig. 3E). Small peaks from highly unsaturated triglycerides were also apparent.

Several of the fish oils contained small amounts of free fatty acids but their contribution to the HPLC traces were negligible. This is because these acids are eluted as two broad peaks covering a restricted section of the chromatograms (15–25 min). Furthermore, the detector response to free fatty acids was approximately one-third that of the triglycerides. Thus, the sharp peaks that were evident on the HPLC chromatograms of the fish oils between 15 and 25 min were not due to free fatty acids.

Analysis of the fractions collected from the Russian, Indian sardine and cod liver oils. The molecular fractions were collected from the Russian, Indian sardine and cod liver oils, as shown in Figure 4. The fatty acid compositional data of the numbered fractions is presented in Tables 3, 4 and 5, respectively. The use of TMAH to methylate the fractions excluded the trace amounts of free fatty acids from the GLC analysis.

The first fraction in all three oils contained only saturated acids which included, for the Indian sardine oil, a significant quantity of 20:0 (3.4%). The saturated acids in Fraction 2 constituted 60.7%, 70.5% and 56.9% of the total for the Russian, Indian sardine and cod liver oils, respectively. The remainder of the acids for this fraction in the Russian and Indian sardine oils were monoenoic, with the percentages obtained for the Indian sardine being very close to those obtained by Laakso *et al.* (21) for Atlantic herring and sand eel. The cod liver oil Fraction 2 included 18:2. The subsequent peak (Fraction 3, Fig. 4C) contained no dienoic acids, only saturated fatty acids and monoenes; 18:1 constituted 35.3% (Table 5). This peak corresponded to triglycerides containing two monoenoic and one saturated fatty acid per glyceride. Thus, 18:1 constituted approximately 30% of the acids from Fraction 3 of the Russian oil (Table 3) and Fraction 4 of the Indian sardine oil (Table 4). Only a trace of 20:1 was present when

TABLE 3

Fatty Acid Composition (% of nominated acids) and Percentage Fatty Acids with Specific Number of Double Bonds for Fractions Obtained Following Silver-Ion HPLC of Russian Fish Oil[a]

Fatty acid	Fraction																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
14:0	14.5	15.7	7.7	2.9	8.0	4.8	9.4	6.3	11.9	7.0	5.7	3.0	5.0	4.9	2.5	—	—	—
15:0	1.2	1.0	Tr	—	—	—	0.6	0.7	0.9	0.4	0.5	Tr	Tr	0.3	—	—	—	—
16:0	79.2	39.1	19.5	7.5	22.4	14.3	23.7	20.7	30.0	19.4	15.2	6.3	13.3	12.2	8.1	5.5	—	—
16:1	—	8.5	12.0	16.9	8.3	12.9	6.1	7.3	3.1	5.5	8.6	8.3	3.9	2.8	4.3	4.1	—	—
16:2	—	—	—	0.9	7.0	7.4	0.4	—	—	—	0.6	1.7	—	—	—	—	—	—
16:3	—	—	—	—	—	3.0	2.9	—	—	Tr	Tr	1.3	4.3	0.6	Tr	Tr	Tr	Tr
16:4	—	—	—	—	—	—	6.5	2.9	Tr	Tr	0.2	1.1	3.8	4.3	0.7	Tr	Tr	3.8
18:0	5.1	4.9	2.1	1.0	3.4	Tr	3.5	3.6	4.5	2.4	2.7	0.9	2.7	2.1	1.8	1.4	—	—
18:1	—	16.5	28.1	36.1	20.3	31.4	13.2	14.1	5.6	14.4	13.0	17.1	8.3	4.3	6.4	9.7	7.0	Tr
18:2	—	—	—	2.3	16.7	18.2	1.9	Tr	Tr	0.6	1.0	3.2	3.2	0.3	Tr	1.4	Tr	—
18:3	—	—	—	—	—	—	5.6	Tr	—	Tr	Tr	1.5	5.7	1.4	Tr	Tr	Tr	Tr
18:4	—	—	—	—	—	—	6.9	14.9	3.4	0.7	0.4	1.7	9.4	8.3	2.6	1.6	7.6	6.4
20:0	—	—	—	—	—	—	Tr	—	Tr	—	0.2	—	—	—	—	—	—	—
20:1	—	7.6	15.7	16.8	8.9	Tr	6.1	7.4	2.1	6.6	6.1	8.0	Tr	1.9	2.3	3.3	Tr	—
20:4[b]	—	—	—	—	—	—	6.4	6.3	1.1	Tr	Tr	1.7	6.3	5.5	0.7	Tr	Tr	2.2
20:5	—	—	—	—	—	—	—	10.7	30.4	32.5	14.8	7.9	17.9	35.1	36.8	26.9	35.7	53.0
22:1	—	6.7	14.9	15.6	5.1	8.0	6.9	5.2	2.9	4.3	6.0	6.3	2.7	1.5	1.6	Tr	10.8	Tr
21:5	—	—	—	—	—	—	—	—	0.9	1.3	0.9	Tr	Tr	1.3	1.5	Tr	Tr	1.8
22:5	—	—	—	—	—	—	—	—	3.2	4.2	3.2	1.2	3.0	4.7	5.2	3.3	Tr	5.4
22:6	—	—	—	—	—	—	—	—	—	0.8	21.0	28.8	10.5	8.4	25.3	42.8	38.9	27.5
23:0[c]	224.4	103.6	114.4	217.5	283.3	209.5	253.8	223.6	231.3	224.8	240.0	235.4	228.1	229.5	217.8	209.2	143.2	202.4
S	100.0	60.7	29.3	11.4	33.8	19.1	37.2	31.3	47.3	29.2	24.3	10.2	21.0	19.5	12.4	6.9	—	—
M	—	39.3	70.7	85.4	42.6	52.3	32.3	34.0	13.7	30.8	33.7	39.7	14.9	10.5	14.6	17.1	17.8	Tr
Di	—	—	—	3.1	23.7	25.6	2.3	Tr	Tr	0.6	1.6	4.9	3.2	0.3	Tr	1.4	Tr	—
Tri	—	—	—	—	—	3.0	8.5	Tr	—	Tr	Tr	2.8	10.0	2.0	Tr	Tr	Tr	Tr
Tet	—	—	—	—	—	—	19.8	24.1	4.5	0.7	0.6	4.5	19.5	18.1	4.0	1.6	7.6	12.4
P	—	—	—	—	—	—	—	10.7	34.5	38.0	18.9	9.1	20.9	41.1	43.5	30.2	35.7	60.2
H	—	—	—	—	—	—	—	—	—	0.8	21.0	28.8	10.5	8.4	25.3	42.8	38.9	27.5

[a]The percentages of the saturated (S), monoenoic (M), dienoic (Di), trienoic (Tri), tetraenoic (Tet), pentaenoic (P) and hexaenoic (H) acids were summed to assess the overall double bond distribution in the triglycerides. Tr, trace.

[b]Includes the n-6 and n-3 isomers.

[c]Area units detected for 23:0 added as a standard to assess recoveries. Fractions 2 and 3 were diluted by a factor of 2.

the total fatty acid composition was determined for the Indian sardine oil, but this fatty acid was measurable in Fraction 4 and one other fraction.

The introduction of more highly unsaturated fatty acids resulted in a loss in base-line resolution and the presence of unresolved complex mixtures. This second phase of the chromatogram was preceded, in all cases, by a sharp peak (Fraction 5, Russian and cod liver oils; Fraction 6, Indian sardine oil). The triglycerides from these fractions contained between 20 and 25% dienoic acids (16:2 and 18:2), while approximately one-third of the acids were saturated and approximately 40% were monoenoic. A large proportion of the triglycerides were probably in the form saturated/monoenoic/dienoic (exact positions not stated). The cod liver oil fraction 5 also contained 16:3 and 16:4. Fraction 6 of this oil (Fig. 4C, Table 5) did not contain these acids. Instead, it contained approximately two-thirds monoenes and one-third dienes. Trienoic acids were present in Fraction 6 of the Russian oil (Fig. 4A, Table 3) and Fraction 7 of the Indian sardine oil (Fig. 4B, Table 4), 16:3 being the major trienoic acid.

A greater variation in peak distribution was observed for peaks which eluted after 27 min. This was a result of the difference in fatty acid composition of the oils. Fractions 7–12 of the Russian oil (Fig. 4A, Table 3) all contained approximately one-third monoenoic acids with the exception of Fraction 9. An increasing range of polyenoic acids was evident in these fractions including 20:5 (from Fraction 8) and 22:6 (from Fraction 10). The average number of double bonds in each triglyceride molecule was 6.9, 7.8 and 8.8 for Fractions 10, 11 and 12, respectively. These fractions also contained the widest range of fatty acids. More detailed examination of these triglycerides requires complementary analysis as illustrated by Laakso and Christie (22). Fraction 8 contained the highest proportion of tetraenoic acids of any fraction. When the percentage tetraenoic acid was plotted against the fraction number, two distinct peaks were observed with maxima at Fraction 8 and Fraction 13. Between these maxima, the percentage decreased to practically zero (Fig. 5). This phenomenon was observed for all other acids when grouped according to the number of double bonds. The pentaenoic acids peaked at Fractions 10 and 15 while DHA peaked at Fractions 12 and 16. In both cases the intermediate values did not drop as low as was observed for the tetraenoic acids. The percentage constituted by the tetraenoic and pentaenoic acids rose, as if toward a third maximum, going from Fraction 16 to 18. Such a maximum was observed for the dienoic acids.

From Fraction 15 onward, the combined percentages for the pentaenoic and hexaenoic acids were greater than 68%. Thus, all these triglycerides contained a minimum of two fatty acids, each of which contained at least five double bonds. Fraction 18 contained only traces of fatty acids

TABLE 4

Fatty Acid Composition (% of nominated acids) and Percentage Fatty Acids with Specific Number of Double Bonds for Fractions Obtained Following Silver-ion HPLC of Indian Sardine Oil[a]

Fatty acid	Fraction																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
14:0	28.3	19.7	21.6	10.8	12.5	11.5	15.0	11.4	14.6	11.0	16.0	8.8	7.8	6.2	5.7	5.6	3.4	1.6	Tr
15:0	3.0	1.7	—	0.8	1.2	Tr	Tr	Tr	1.2	1.2	1.3	0.9	0.8	0.7	Tr	Tr	Tr	Tr	—
16:0	54.1	40.4	58.0	22.3	25.0	23.0	29.9	22.7	28.5	25.2	29.5	19.0	16.3	13.2	11.6	11.1	10.5	5.0	Tr
16:1	—	14.5	20.5	30.0	21.2	18.6	9.3	18.1	6.8	12.0	2.3	14.2	11.3	7.6	5.6	2.4	5.6	4.7	Tr
16:2	—	—	—	—	4.9	10.9	4.5	Tr	1.4	0.6	0.5	Tr	2.4	2.3	0.9	—	Tr	1.6	—
16:3	—	—	—	—	—	—	15.4	19.3	7.5	1.4	0.8	—	1.5	8.1	4.9	Tr	0.8	2.3	2.7
16:4	—	—	—	—	—	—	—	Tr	6.4	3.9	0.4	0.9	0.6	2.1	4.4	4.6	Tr	1.7	2.7
18:0	11.2	7.4	—	3.2	4.9	4.0	6.3	4.8	5.8	5.5	5.0	4.1	3.3	2.9	2.6	2.7	3.4	1.9	—
18:1	—	14.8	Tr	31.0	22.1	18.8	7.9	14.8	5.4	8.4	2.7	9.8	9.4	4.1	4.0	—	3.5	6.6	2.2
18:2	—	—	—	—	7.1	13.3	5.9	2.8	1.8	0.7	0.5	Tr	2.1	2.4	0.6	—	Tr	1.5	—
18:3	—	—	—	—	—	—	2.6	6.1	1.7	Tr	Tr	—	0.3	1.6	1.3	—	Tr	Tr	Tr
18:4	—	—	—	—	—	—	—	—	3.4	12.1	2.2	0.8	1.0	3.5	7.7	3.6	1.7	3.2	4.9
20:0	3.4	1.3	—	0.6	1.2	Tr	1.6	Tr	1.4	0.9	0.8	—	0.5	—	Tr	—	Tr	—	—
20:1	—	Tr	—	1.3	Tr	Tr	1.6	—	—	Tr	Tr	—	Tr	—	—	—	—	—	—
20:4	—	—	—	—	—	—	—	—	14.1	10.9	1.2	0.6	1.1	3.9	9.8	1.7	0.9	1.7	2.5
20:5	—	—	—	—	—	—	—	—	—	6.2	33.5	27.7	19.2	22.5	27.8	55.5	39.0	28.2	51.6
22:1	—	—	Tr	—	Tr	Tr	—	—	—	Tr	—	—	Tr	—	—	—	—	—	—
21:5	—	—	—	—	—	—	—	—	—	—	1.3	1.1	0.8	0.8	1.0	1.5	1.7	Tr	1.9
22:5	—	—	—	—	—	—	—	—	—	Tr	2.1	2.3	1.6	1.7	2.0	4.3	3.7	2.3	4.4
22:6	—	—	—	—	—	—	—	—	—	—	—	10.0	20.0	16.1	10.0	7.1	25.9	37.6	27.0
23:0[b]	263.5	117.3	229.3	297.2	288.0	274.4	238.4	257.6	310.4	297.8	227.0	169.0	198.9	247.7	216.8	101.8	235.8	230.1	219.8
S	100.0	70.5	78.6	37.7	44.8	38.5	52.8	38.9	51.5	43.8	52.6	32.8	28.7	23.0	19.9	19.4	17.3	8.5	Tr
M	—	29.3	20.5	62.3	43.3	37.4	18.8	32.9	12.2	20.4	5.0	24.0	20.7	11.7	9.6	2.4	9.1	11.3	2.2
Di	—	—	—	—	12.0	24.2	10.4	2.8	3.2	1.3	1.0	Tr	4.5	4.7	1.5	—	Tr	3.1	—
Tri	—	—	—	—	—	—	18.0	25.4	9.2	1.4	0.8	Tr	1.8	9.7	6.2	Tr	0.8	2.3	2.7
Tet	—	—	—	—	—	—	—	—	23.9	26.9	3.8	2.3	2.7	9.5	21.9	9.9	2.6	6.6	10.1
P	—	—	—	—	—	—	—	—	—	6.2	34.8	31.1	21.6	25.0	30.8	61.3	44.4	30.5	57.9
H	—	—	—	—	—	—	—	—	—	—	—	10.0	20.0	16.1	10.0	7.1	25.9	37.6	27.0

[a]The percentages of the saturated (S), monoenoic (M), dienoic (Di), trienoic (Tri), tetraenoic (Tet), pentaenoic (P) and hexaenoic (H) acids were summed to assess the overall double bond distribution in the triglycerides. Tr, trace.

[b]Area units detected for 23:0 added as a standard to assess recoveries. Fraction 2 was diluted by a factor of 2.

with less than four double bonds. In addition, the percentages obtained for the pentaenoic/hexaenoic acids indicated that there were triglycerides substituted at all three positions by fatty acids with a minimum of five double bonds.

The triglyceride fractions from the Indian sardine oil containing polyenoic acids (Fractions 8 to 19; Fig. 4B, Table 4) were derived from more distinct peaks. Fraction 8 contained the highest percentage of 16:3 of any fraction from the three oils. Fraction 9 contained the first of the quantifiable tetraenoic acids (16:4, 18:4 and 20:4). Fraction 10 was a composite fraction, which contained a small proportion of EPA.

Fractions 11–13 (Fig. 4B) were collected from three major composite peaks. Fraction 11 contained 34.8% pentaenoics, primarily EPA, and 52.6% saturated fatty acids. Thus, the bulk of the triglycerides were saturated/pentaenoic. Approximately one-third of the acids from Fraction 12 were saturated, and an equivalent proportion contained five double bonds. In this oil, DHA was first detected in Fraction 12.

From Fraction 13 onward, of the sardine oil, there was a steady decrease in the percentage of saturated acids. The percentages of pentaenoic acids attained a maximum in Fraction 16. All triglycerides from Fraction 16 onward contained two acids with at least five double bonds per acid. This was the same as was observed for the Russian oil. Fraction 19 contained measurable amounts of 18:1, 16:3 and trace amounts of the saturated acids 14:0 and 16:0.

Plotting the combined area percentages for the acids containing a specific number of double bonds against the fraction number again gave discrete peaks except for pentaenoic acids.

The earlier-eluting cod liver oil triglycerides gave sharper, more intense peaks relative to the corresponding fractions in the two preceding fish oils. Examples of this were the peaks which comprised Fractions 7, 8 and 9 (Fig. 4C). The largest percentage for 18:3 obtained in a fraction of any of the oils was found in cod liver oil Fraction 9. Fraction 10 was a low broad peak. The fatty acid composition was approximately one-third saturated fatty acid, one-third monoenes with the remaining third comprised of di-, tri- and tetraenoic acids.

Cod liver oil EPA first appeared in Fraction 11, where it comprised 4.4% of the fatty acids. The DHA was first identified in Fraction 13 and comprised 26.6% of Fraction 14. Fraction 15 was characterized by a large percentage of tetraenoic acids.

The saturated acid content diminished markedly over the last three fractions of the Russian fish oil and Indian sardine oil. The content of both saturated and monoenoic acids was sustained in the cod liver oil such that there was a significant population of both groups of fatty acids in the final fraction. This was probably due to the influence of a large peak present at the start of Fraction 18

TABLE 5

Fatty Acid Composition (% of nominated acids) and Percentage Fatty Acids with Specific Number of Double Bonds for Fractions Obtained Following Silver-Ion HPLC of Seven Seas Cod Liver Oil[a]

Fatty acid	Fraction																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
14:0	43.5	13.6	8.1	2.3	6.7	0.9	11.8	4.8	4.9	10.8	5.0	10.6	6.7	4.8	4.2	4.2	2.3	2.1
15:0	—	1.7	0.5	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	0.7	0.5	0.4	Tr	Tr	Tr	Tr
16:0	56.5	35.0	20.5	6.2	21.9	3.4	30.1	14.6	12.2	24.0	14.7	26.8	17.2	11.3	11.0	10.6	7.1	5.3
16:1	—	9.1	12.3	12.9	7.2	4.7	4.5	5.8	4.9	6.8	8.8	5.1	8.1	9.4	4.3	2.8	4.3	2.7
16:2	—	—	—	Tr	2.6	1.6	Tr	Tr	Tr	Tr	Tr	Tr	0.3	1.1	0.8	Tr	Tr	Tr
16:3	—	—	—	—	2.2	Tr	2.8	3.4	1.3	Tr	Tr	Tr	Tr	0.5	1.9	Tr	Tr	Tr
16:4	—	—	—	—	6.0	—	—	—	3.5	6.0	2.2	Tr	Tr	0.5	4.0	1.9	Tr	1.7
18:0	—	6.6	3.1	1.1	4.9	1.2	8.8	2.9	2.2	3.2	2.7	4.7	3.1	2.2	2.2	3.1	1.8	1.4
18:1	—	19.5	35.3	56.9	28.4	54.3	26.2	27.2	35.4	13.6	21.6	9.1	15.4	16.8	8.0	8.4	7.6	7.1
18:2	—	8.5	—	1.3	17.6	28.7	9.7	25.2	7.4	3.5	7.1	0.9	1.2	2.2	1.6	Tr	Tr	1.1
18:3	—	—	—	—	Tr	—	3.0	10.3	17.4	2.6	6.5	Tr	0.8	1.3	3.4	Tr	Tr	0.9
18:4	—	—	—	—	—	—	—	—	1.5	10.4	12.1	4.9	0.8	0.9	8.2	6.7	2.7	3.7
20:0	—	Tr	Tr	—	—	—	—	Tr	Tr	—	—	Tr	0.3	Tr	—	—	—	—
20:1	—	3.6	11.6	12.0	Tr	4.1	3.1	3.9	3.6	4.3	4.8	3.3	5.0	5.2	2.2	Tr	1.8	0.9
20:4	—	—	—	—	—	—	—	—	3.3	8.0	5.5	1.4	Tr	0.7	5.8	3.0	Tr	2.0
20:5	—	—	—	—	—	—	—	—	—	—	4.4	26.8	22.8	9.4	22.4	39.9	36.0	27.7
22:1	—	2.5	8.6	7.3	2.5	1.1	Tr	1.9	2.4	6.9	4.4	2.5	3.9	3.6	2.5	1.2	1.0	0.8
21:5	—	—	—	—	—	—	—	—	—	—	—	0.7	1.0	0.5	0.8	1.4	1.4	1.0
22:5	—	—	—	—	—	—	—	—	—	—	—	2.7	4.4	2.6	3.3	5.6	5.9	3.7
22:6	—	—	—	—	—	—	—	—	—	—	—	—	8.5	26.6	13.4	11.2	28.1	37.8
23:0[b]	231.0	132.8	125.9	129.0	107.9	250.0	284.5	280.0	267.4	273.0	265.9	236.2	243.2	243.4	232.1	230.1	268.4	247.5
S	100.0	56.9	32.2	9.6	33.5	5.5	50.7	22.3	19.3	38.0	22.4	42.8	27.8	18.7	17.4	17.9	11.2	8.8
M	—	34.7	67.8	89.1	38.1	64.2	33.8	38.8	46.3	31.6	39.6	20.0	32.4	35.0	17.0	12.4	14.7	11.5
Di	—	8.5	—	1.3	20.2	30.3	9.7	25.2	7.4	3.5	7.1	0.9	1.5	3.3	2.4	Tr	Tr	1.1
Tri	—	—	—	—	2.2	Tr	5.8	13.7	18.7	2.6	6.5	Tr	0.8	1.8	7.7	Tr	Tr	0.9
Tet	—	—	—	—	6.0	—	—	—	8.3	24.4	19.8	6.3	0.8	2.1	18.0	11.6	2.7	7.4
P	—	—	—	—	—	—	—	—	—	—	4.4	30.2	28.2	12.5	26.5	46.9	43.3	32.4
H	—	—	—	—	—	—	—	—	—	—	—	—	8.5	26.6	13.4	11.2	28.1	37.8

[a]The percentages of the saturated (S), monoenoic (M), dienoic (Di), trienoic (Tri), tetraenoic (Tet), pentaenoic (P) and hexaenoic (H) acids were summed to assess the overall double bond distribution in the triglycerides. Tr, trace.

[b]Area units detected for 23:0 added as a standard to assess recoveries. Fractions 2 and 3–5 were diluted by a factor of 1.5 and 2, respectively.

and the presence of the tail end of a small unresolved complex mixture.

Despite the differences between the cod liver oil and the other two oils, plotting the combined area percentage of the acids with equivalent number of double bonds against fraction number gave similar results with distinct peaks for the individual double bond types.

DISCUSSION

In this study, the fatty acid and triglyceride distributions of industrial, retail and laboratory-extracted fish oils have been investigated using a combination of GLC and Ag^+-HPLC. The objective was to study the range and variations in molecular species of triglyceride present in such oils and to investigate the potential of utilizing Ag^+-HPLC for assessing the nature of fish oils. No attempt was made to isolate the triglycerides free of the trace amounts of wax esters, free fatty acids or sterols found to be present in the fish oils by TLC. The emphasis was on utilizing the oil directly. The potential for interference from these compounds was assessed by carrying out HPLC analysis on appropriate standards. The saturated wax ester, palmityl palmitate, was eluted just before the trisaturated triglyceride peak; the two peaks were not totally resolved. Monounsaturated wax esters, typified by oleyl palmitate, were located between the trisaturated and monounsaturated triglyceride peaks. Oleyl oleate had a retention time greater than for triglycerides which contained two monoenoic acids per molecule. Orange roughy oil, which contained mainly wax esters, gave essentially two peaks when analyzed by Ag^+-HPLC. The retention times were the same as those of oleyl palmitate and oleyl oleate. Thus, the wax esters are grouped according to degree of saturation and the retention times were independent of chain length. Close examination of the trisaturated triglyceride peak in the chromatograms from Issac Spencer and Sun'n'Sea cod liver oils revealed a shoulder on the leading edge of the peak. This was probably due to the saturated wax esters. There was no evidence for saturated, or any other, wax esters in the oils chosen for fractionation.

From the results obtained during this study and those of Laakso *et al.* (21), it was apparent that the triglyceride composition of all fish oils is extremely complex. The composition of the triglycerides range from those containing only saturated acids to those containing up to at least 16 double bonds.

Although there is insufficient information from this study to conclude whether or not Ag^+-HPLC can be used to fingerprint specific fish oils, there is good evidence that the body oils examined in this work can be categorized as belonging to one of two groupings. The mackerel oil (admittedly from one fish), herring and capelin oils were similar to each other but different from menhaden, South African anchovy and Indian sardine oils. The former were

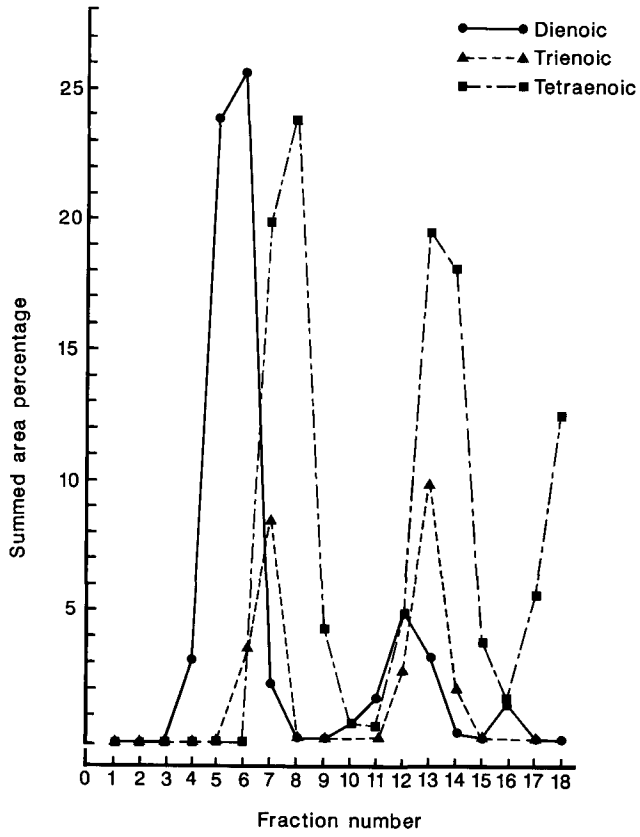

FIG. 5. Plot of summed area percentage of fatty acids with equivalent number of double bonds against fraction number for dienoic, trienoic and tetraenoic acids from the Russian fish oil derived fractions.

characterized by a large peak between 11 and 13 min with a second, smaller, peak between 17 and 21 min. These two peaks were typified by a high proportion of monoenoic acids. Oils where these peaks were very small or absent (Indian sardine, South African anchovy and menhaden oils) contained a very small proportion of 20:1 and 22:1. It can also be seen from the triglyceride profiles that peaks eluting after 50 min, which correspond to the polyunsaturated triglycerides, are either absent or present in trace amounts for mackerel, herring and capelin, whereas significant amounts are to be found in the menhaden, South African anchovy and Indian sardine profiles.

The Chilean oil gave an HPLC profile more akin to the latter group of oils whereas that of the Russian oil appeared to encompass both. The distribution of the 20:1 and 22:1 in the Chilean and Russian oils followed the expected pattern. As far as the liver oils were concerned, the Isaac Spencer cod liver oil was identified as being atypical from its triglyceride pattern. This interpretation was reinforced by its unusual fatty acid composition.

From the results obtained in this study, it is apparent that Ag^+-HPLC can be used to characterize fish oils. The distinctive peak shapes and triglyceride distribution of the fish oil profiles contrast markedly with those from plant oils. Thus, Ag^+-HPLC can be used to rapidly differentiate between triglycerides from plant and fish oils or to check for adulteration.

The method, as presented, was reproducible, but reconditioning of the column or a change of column required a reevaluation of the solvent gradients. In addition, accurate and reproducible retention times for the early eluting peaks were only achieved if the time permitted for reequilibration of the column with the starting solvent remained constant. This favored the use of an autosampler as did the time of analysis. In addition, after an overnight stop a blank run had to be performed before any triglyceride analysis could be carried out.

Marine 25 was described as a concentrate. This fish oil contained a high proportion of 20:5 and 22:6, but triglycerides which contained three saturated fatty acids or two saturated fatty acids and one monoenoic fatty acid per triglyceride were still present. From the work carried out in this study, it was apparent that the triglyceride distributions follow a similar pattern for fish oils. A method which removed from a fish oil all the triglycerides with structures equivalent to those eluted in the first 29 min of a typical chromatogram would yield an enriched oil, with maximal amounts of 20:5 and 22:6 being retained. The residual product should also have some commercial value. Chromatography with Ag^+-HPLC could be utilized to assess the suitability of a fish oil for such an enrichment process. This line of research is being actively pursued in this laboratory.

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Lipid Composition and Mitochondrial Respiration in Warm- and Cold-Adapted Sea Bass

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The response to cold of liver and heart membrane lipid composition and mitochondrial respiration in reared sea bass (*Dicentrarchus labrax* L.) was investigated. Fish acclimation was followed during the natural seasonal cycle from August to March. The data on the fatty acid composition of liver and heart polar lipids and on total lipids of liver mitochondria and microsomes did not indicate any increase in unsaturation in response to cold. The enzyme complexes of the liver and heart mitochondrial respiratory chain showed a repeated negative compensation for cold acclimation. The constancy of the break in the Arrhenius plot of liver cytochrome oxidase (EC 1.9.3.1.) was consistent with the lack of homeoviscous adaptation of membrane lipids. A thermoadaptive strategy based on the reduction of sea bass metabolic activity is suggested. *Lipids 27,* **371-377 (1992).**

Various physiological and biochemical mechanisms have evolved in poikilotherms to counter the effects of thermal variation on physical properties of cells and body fluids. A number of studies have been carried out to identify the temperature-activated mechanisms involved in maintaining homeostasis. Changes in the unsaturation of membrane lipids have generally been implicated in membrane viscotropic regulaton and thus in the modulation of membrane-bound enzyme activities (1-5). However variations in the membrane cholesterol content (6) and in the ratio of phospholipid classes (3,7), interactions between the polar heads of phospholipids (8) and/or lipids and proteins (8,9), as well as direct temperature effects on protein chains (2), have been shown to contribute to homeoviscous adaptation. These studies have been mainly carried out on winter-active freshwater teleosts. In some poikilotherms, the slowing of life activities, similar to that characteristic for hibernating mammals, was described as another adaptational strategy (2,10).

The purpose of our study was to examine whether behavioral adaptation in the sea bass (*Dicentrarchus labrax* L.) is accompanied by changes in membrane lipid composition and/or by variations in the activity of membrane-bound enzymes, such as mitochondrial respiratory complexes. The choice of the sea bass was based on the following observations: below 10°C, this species strongly reduces motility and stops eating (11); its marine origin implies reduced capability to synthesize polyunsaturated fatty acids (PUFA) (12). Our experiments were carried out following the seasonal cycle to obtain more reliable results than one could obtain by artificial, season-independent cold-acclimation studies. Thus, our experimental design reproduced the natural conditions, fast included, by overwintering sea bass in Northern Italy (11).

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate; FA, fatty acids; GLC, gas-liquid chromatography; LCA, long-term cold-adapted; MUFA, monounsaturated fatty acids; PL, phospholipids; PUFA, polyunsaturated fatty acids; SCA, short-term cold-adapted; SFA, saturated fatty acids; TL, total lipids; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylendiamine dihydrochloride; Tris, Tris-(hydroxymethyl)aminomethane; WA, warm-adapted.

MATERIALS AND METHODS

Reagents. Solvents, acid-washed Chromosorb W 60-80 mesh and diethyleneglycolsuccinate were purchased from Carlo Erba (Milan, Italy). Silver nitrate and Tris were obtained from Merck (Darmstadt, Germany). Silicic acid, 100 mesh and 14% boron trifluoride in anhydrous methanol were purchased from Mallinkrodt (St. Louis, MO) and from BDH, Laboratory Chemical Division (Poole, United Kingdom), respectively. Fatty acid-free bovine serum albumin (BSA), sodium ethylenediaminetetraacetate (Na_2EDTA), respiratory substrates, and inhibitors were from Sigma Chemical Co. (St. Louis, MO). Standards for fatty acid analyses were obtained from Sigma and from Applied Science Laboratories Inc. (State College, PA). All chemicals were reagent grade. Quartz-double distilled water was used for all solutions in the preparation of subcellular fractions and in the mitochondrial respiratory activity tests.

Animals and diets. Adult sea bass (*Dicentrarchus labrax* L.) weighing about 100 g, reared in a commercial hatchery in Valle Campo-Comacchio (Ferrara, Italy), were used. Fish were fed a pelleted diet to which lipids had been added as oil mixture (cod liver oil/raw linseed oil/grape seed oil/corn oil, 2:1:1:1, by vol) amounting to 10% (13). Total dietary total lipid content was slightly higher (11.2%, w/w) due to the lipid content of other dietary components. The fatty acid pattern of the diet is shown in Table 1. Other details of diet composition were reported by Corti *et al.* (13).

Experimental design. As shown in Figure 1 fish were kept at approximately 22°C, from August until mid-October (warm-adapted or WA) and fed to satiation (2.5% of wet body weight/day). Following the seasonal lowering of environmental temperature, the water temperature gradually decreased to 10°C. Food consumption gradually decreased with temperature and became undetectable. The daily ration was therefore gradually lowered to 0.5% of wet body weight and maintained until the end of the experiment. Fish sacrificed in December, after a month of acclimation at temperatures around 10-11°C to test the short-term cold adaptation to the threshold temperature of food uptake among sea bass (11), were defined as short-term cold-adapted (SCA). Fish were then kept at approximately 7°C until March to test the long-term adaptation at a temperature similar to that faced by reared sea bass during winter in Northern Italy (7). This adaptation was performed under two conditions as follows. Fish were randomly divided into two groups. The first group was kept in seawater tanks and diet was provided (Expt. 1). In parallel, the second group was maintained at the same

TABLE 1

Fatty Acid Composition of the Diet[a]

Fatty acid	wt/%
14:0	1.5
16:0	10.4
16:1	1.7
18:0	3.4
18:1	33.4
18:2n-6	31.7
18:3n-3	5.4
20:1n-9	3.1
20:4n-6	0.2
22:1n-9	2.6
20:5n-3	2.0
22:6n-3	3.4
Others[b]	4.6
ΣSFA	17.3
ΣMUFA	39.4
ΣPUFA	43.3
Σn-3	11.4
Σn-6	31.9
I	1.53
I × 100/ΣSFA	8.84
n-3/n-6	0.36

[a] Acronyms are listed in Materials and Methods. Values are means of at least 3 determinations.

[b] Sum of 12:0, 13:0, 15:0, *iso*-16:0, 17:0, *iso*-18:0, 19:1, 18:3n-6, 18:4n-3, 20:3n-6, 20:4n-3, 22:5n-3, 24:0 and 24:1.

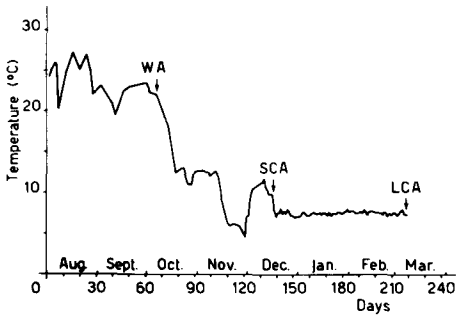

FIG. 1. Temperature regimen during the experiment. Samplings are indicated by arrows.

temperature in synthetic-seawater tanks, but was not fed (Expt. 2). Since at this temperature sea bass usually do not eat (11), the two experiments were aimed at ascertaining whether any differences exist between fasting (Expt. 1) and starved (Expt. 2) sea bass. Data from both experiments were not significantly different; therefore, the results from long-term cold-acclimated (LCA) fish are reported as averages of Expts. 1 and 2. The water temperature in the tanks was measured at the same hour each day during the entire experiment. Fish mortality, which was calculated as percentage of the initial number at the end of each acclimation period, was about 0.9% up to December and nil thereafter (Viviani, R., personal communication).

Sampling procedures. Fish were randomly selected and killed at the end of each experimental period. Samples consisted of 80 WA, 40 SCA and 80 LCA fish (60 from Expt. 1 and 20 from Expt. 2). Liver and heart were removed immediately after death. Part of each tissue was stored in the dark under nitrogen at −20°C for lipid analysis. Another portion was immediately used for the preparation of subcellular fractions.

Preparation of the mitochondrial and microsomal fractions. In each preparation, the liver and heart from 10–20 animals were repeatedly rinsed in ice-cold medium A (0.25 M sucrose, 5 mM sodium ethylenediaminetetraacetate (Na_2EDTA), 16.5 mM Tris-HCl, pH 7.4) and homogenized with an Ultraturrax in medium B (0.25 M sucrose, 1 mM Na_2EDTA, 24 mM Tris-HCl, 0.5 mg/mL fatty acid-free bovine serum albumin (BSA), pH 7.6). The mitochondrial fraction was obtained by differential centrifugation using the method of Lyons and Raison (14) modified as follows. The homogenate was centrifuged at 750 × *g* for 10 min, and then the supernatant was filtered through a double layer of gauze (30 mesh) and centrifuged at 10,000 × *g* for 10 min. The mitochondrial pellet was resuspended in medium B and centrifuged at 13,000 × *g* for 10 min. Part of the sample was frozen in liquid nitrogen for lipid analysis, and another part was immediately used for the respiratory tests. The post-mitochondrial supernatant from the liver was further centrifuged at 90,000 × *g* for 90 min. The pellet was resuspended and twice washed by centrifuging at the same speed for 40 min. The resulting microsomal fraction was stored in liquid nitrogen until use. All steps were carried out at 0–4°C. Protein in the various fractions was evaluated by the biuret method (15). The purity of microsomal and mitochondrial fractions was routinely tested by determining (Na^+, K^+)-ATPase (EC 3.6.1.3) and cytochrome oxidase (EC 1.9.3.1) as enzymatic markers for plasma membrane and mitochondria, respectively. The negligible specific activity of (Na^+, K^+)-ATPase in the mitochondrial fraction and of the cytochrome oxidase in microsomes indicated no appreciable mutual contamination of the fractions under study.

Determination of mitochondrial respiratory activities. The respiratory activity of liver and heart mitochondria was evaluated polarographically with a Clark electrode using a Yellow Springs Instrument Model 53 oxygen monitor (Yellow Springs, OH). The three sites of oxidative phosphorylation were tested at 20°C by using as substrates, respectively, glutamate, succinate and ascorbate + *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine dihydrochloride (TMPD) under the conditions previously described (16). The respiratory control ratio (RCR) and adenosine diphosphate (ADP):Oxygen (O) ratio were calculated as described by Estabrook (17). The Arrhenius plot of the liver cytochrome oxidase system was drawn by plotting the state 3 respiratory activity data using ascorbate + TMPD as substrate in the temperature range of 3–40°C as reported by Borgatti *et al.* (18). The above assay was not carried out on heart because of insufficient mitochondrial material.

Lipid analyses. Lipids were extracted according to Folch *et al.* (19) from each liver mitochondrial and microsomal preparation containing 100–300 mg protein, from 1–5 g liver and heart pooled from 5 fish, and from 5 g of the diet. Total lipids (TL) were determined gravimetrically. Phospholipids (PL) were quantitatively evaluated by colorimetric determination of phosphorus (20) according to the method of Bartlett (21), as modified by Marinetti (22). Tissue lipid extracts were separated into neutral lipids and

polar lipids according to Marks *et al.* (23). Activated silicic acid in the maximal ratio of 1 g per 50 mg PL was added to total lipids. After removal of neutral lipids by triplicate extraction with diethyl ether, polar lipids were obtained by triplicate extraction with methanol. A complete separation was obtained. The saponification of TL and polar lipids, the extraction of the fatty acids (FA), and the methylation using 14% BF_3 in methanol were carried out as previously described (24). Methyl esters of FA were analyzed by gas-liquid chromatography (GLC) and also by chromatography on silicic acid columns impregnated with 25% $AgNO_3$ and by catalytic hydrogenation. GLC was done on a Dani (Monza, Italy) 3600 gas chromatograph using a glass column (2 m × 4 mm) filled with acid-washed Chromosorb W 60–80 mesh and 20% (w/w) diethyleneglycolsuccinate. The carrier gas was nitrogen. The GLC operating conditions and the methods of identification of FA were reported previously (25). Methyl esters of FA separated by GLC are given as weight percentages of total FA. The unsaturation index (I) and the ratio I × 100/Σ saturated fatty acids (SFA) were calculated according to Bloj *et al.* (26).

Statistics. Results are presented as mean ± standard error (SE). Statistical significance of differences between differently acclimated groups was determined by the Student's t-test.

RESULTS AND DISCUSSION

Effect of cold-acclimation on lipid composition. Changes in lipid class composition have been reported as one of the possible responses to temperature lowering (27,28). The content of total lipids (TL) and phospholipids (PL) of liver, heart and liver mitochondria and microsomes, shown in Table 2, indicates that only in the liver TL and PL levels were changed by cold exposure. The observed increase of liver TL in SCA probably reflects fat storage to face the winter. This would be consistent with previous studies which have shown that eel accumulates triglycerides as energy source before fasting for the winter (28). A TL increase was found by Roche and Pérès (27) in cold-acclimated *Dicentrarchus labrax* fed during the entire experiment. However, other cold acclimation studies (29) that were carried out under conditions of forced starvation and independent of season showed no TL variation. The PL increase in LCA is probably related to the consumption of previously stored neutral lipids. Other studies have indicated a widespread tendency of PL to increase after long-term cold-exposure (at least one month) (27,28).

No variations in TL and PL levels are apparent in heart and liver subcellular fractions consistent with the lack of fat storage function of this tissue. The constancy of TL and PL levels in liver mitochondrial and microsomal fractions is also in line with literature reports on cold acclimated teleosts (6,9,30).

The analysis of fatty acid composition was focused on liver and heart polar lipids and on total lipids of liver mitochondrial and microsomal fractions.

Liver polar lipids (Table 3) show only little variation related to cold acclimation, namely an increase of 20:5n-3 and thus of Σn-3, and a decrease of 18:1 resulting into a lowering of monounsaturated fatty acids (MUFA). The decrease of MUFA is consistent with that reported for trout liver (31).

TABLE 2

Total Lipid and Phospholipid Content of Liver and Heart and Liver Subcellular Fractions[a]

	n[b]	TL[c]	PL[c]
Liver			
WA	6	30.4 ± 1.0	2.5 ± 0.1
SCA	2	37.1 ± 0.5[d]	2.2 ± 0.1
LCA	4	32.5 ± 2.9	3.2 ± 0.1[e]
Heart			
WA	6	4.0 ± 0.8	1.8 ± 0.1
SCA	2	4.0 ± 0.5	1.6 ± 0.1
LCA	4	3.6 ± 0.8	1.8 ± 0.2
		TL[f]	PL[f]
Liver			
Mitochondria			
WA	6	19.6 ± 1.8	13.4 ± 0.9
SCA	2	18.4 ± 1.2	13.2 ± 2.4
LCA	4	17.1 ± 1.8	10.9 ± 0.5
Microsomes			
WA	6	42.2 ± 4.6	23.3 ± 3.9
SCA	2	40.2 ± 4.4	20.5 ± 1.7
LCA	4	51.9 ± 2.1	28.9 ± 1.2

[a] Acronyms are listed in Materials and Methods.

[b] Number of pools analyzed.

[c] Expressed as wet wt percentages, mean ± SE.

[d] $p \leq 0.05$, [e] $p \leq 0.01$, significance with WA.

[f] Expressed as wt percentages of protein content, mean ± SE.

Heart polar lipids show a rather constant fatty acid composition (Table 3). The observed decreases in 18:0, 18:1 and MUFA and the increase of 22:6n-3 during cold acclimation are insufficient to affect the unsaturation parameters.

When the acclimation temperature is lowered, liver mitochondrial and microsomal FA (Table 4) show a progressive increase in 18:2n-6 and a decrease in 22:6n-3 with a consequent increase in Σn-6 and a decrease in the unsaturation parameters, Σn-3 and the n-3/n-6 ratio. Some of these data are at odds with many literature reports that show an overall increase in unsaturation parameters during cold adaptation (3,4,6,8). In carp liver, cold was shown to promote a decrease in 22:6n-3 and in the n-3/n-6 ratio, which consistent with previous findings was counterbalanced by an increase in n-6 long-chain polyunsaturated fatty acids (PUFA) due to Δ6 desaturation of linoleic acid. Consequently membrane unsaturation increased (7). By contrast, in cold acclimated sea bass, an increase in 18:2n-6, which is not further desaturated and elongated, together with a decrease in 22:6n-3 contributed to lower unsaturation parameters. The high level of 18:2n-6 may have been due to dietary intake which is higher in reared fish than in wild ones (27).

Membrane fatty acid composition has received the most attention as possible site of homeoviscous adaptation (1–4). The lack of an increase in unsaturation parameters during cold adaptation, which is at odds with other literature reports, may have been due to differences in the experimental design of our studies and of those reported earlier. The differences lie in the experimental conditions

TABLE 3

Fatty Acid Composition of Polar Lipids of Liver and Heart[a]

Fatty acid (wt%)	Liver			Heart		
	WA n[b] = 6	SCA n = 2	LCA n = 4	WA n = 6	SCA n = 2	LCA n = 4
14:0	1.8 ± 0.2	1.4 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.4 ± 0.2	0.8 ± 0.2
16:0	20.2 ± 0.2	20.3 ± 0.3	20.9 ± 0.6	22.2 ± 0.5	25.1 ± 0.1	24.0 ± 0.7
16:1	3.4 ± 0.1	2.3 ± 0.1	2.2 ± 0.4	2.5 ± 0.1	2.5 ± 0.1	2.0 ± 0.1
18:0	12.5 ± 0.5	12.3 ± 0.1	11.5 ± 0.5	14.3 ± 0.3	13.6 ± 0.4	13.1 ± 0.1[c]
18:1	22.1 ± 0.9	22.0 ± 0.3	17.1 ± 1.7[d]	27.3 ± 0.2	23.8 ± 0.5[c]	21.7 ± 1.3[c]
18:2n-6	10.2 ± 1.7	11.4 ± 0.1	12.5 ± 0.5	8.0 ± 0.1	10.2 ± 0.2	1.06 ± 0.7
18:3n-3	0.6 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.1
20:1n-9	0.9 ± 0.2	1.5 ± 0.1	1.0 ± 0.1	1.7 ± 0.2	1.5 ± 0.0	1.5 ± 0.2
20:2n-6	0.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	1.4 ± 0.1	1.2 ± 0.1
20:4n-6	2.4 ± 0.2	2.3 ± 0.1	2.6 ± 0.7	1.8 ± 0.3	1.5 ± 0.1	1.1 ± 0.1
20:5n-3	2.7 ± 0.3	3.0 ± 0.1	4.6 ± 0.6[d]	3.3 ± 0.2	2.8 ± 0.1	3.2 ± 0.2
22:6n-3	18.5 ± 2.2	16.6 ± 2.1	20.9 ± 1.8	13.4 ± 1.0	13.1 ± 1.1	18.0 ± 0.9[d]
Others[e]	4.0 ± 0.2	4.9 ± 0.3	4.0 ± 0.2	2.6 ± 0.5	2.3 ± 0.5	1.7 ± 0.4
ΣSFA	37.2 ± 2.0	36.6 ± 0.8	36.0 ± 1.0	38.6 ± 2.3	41.0 ± 1.7	38.6 ± 1.5
ΣMUFA	27.1 ± 0.8	26.9 ± 1.5	21.1 ± 0.7[c]	32.8 ± 1.3	28.8 ± 2.1	26.3 ± 1.5[d]
ΣPUFA	35.7 ± 2.7	36.4 ± 2.3	43.1 ± 3.5	28.6 ± 1.7	30.2 ± 2.0	35.1 ± 1.5
Σn-3	22.3 ± 1.3	21.7 ± 1.9	27.0 ± 1.3[d]	18.1 ± 1.3	17.1 ± 1.7	22.3 ± 1.5
Σn-6	13.4 ± 1.7	14.7 ± 0.7	16.1 ± 1.0	10.8 ± 1.3	13.1 ± 1.2	12.9 ± 1.4
I	1.90 ± 0.07	1.80 ± 0.10	2.13 ± 0.10	1.61 ± 0.13	1.46 ± 0.19	1.87 ± 0.22
I × 100/ΣSFA	5.11 ± 0.22	4.91 ± 0.13	5.90 ± 0.50	4.22 ± 0.23	3.76 ± 0.12	4.93 ± 0.17
n-3/n-6	1.66 ± 0.02	1.48 ± 0.12	1.68 ± 0.02	1.67 ± 0.07	1.31 ± 0.10	1.73 ± 0.09

[a] Acronyms are listed in Materials and Methods.

[b] Number of pools analyzed. Values are means of n pools ± SE.

[c] $p \leq 0.01$, significance with WA; [d] $p \leq 0.05$, significance with WA.

[e] See footnote b of Table 1.

TABLE 4

Fatty Acid Composition of Total Lipids of Liver Mitochondria and Microsomes[a]

Fatty acid (wt%)	Mitochondria			Microsomes		
	WA n[b] = 6	SCA n = 2	LCA n = 4	WA n = 6	SCA n = 2	LCA n = 4
14:0	1.0 ± 0.0	0.9 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
16:0	18.0 ± 0.5	17.3 ± 0.3	19.8 ± 1.2	19.2 ± 0.8	18.3 ± 0.2	20.6 ± 0.6
16:1	2.5 ± 0.1	2.7 ± 0.1	3.9 ± 0.3[c]	2.9 ± 0.1	2.8 ± 0.1	3.2 ± 0.1
18:0	8.8 ± 0.2	9.1 ± 0.1	8.5 ± 0.2	10.3 ± 0.2	10.4 ± 0.5	8.5 ± 0.1
18:1	15.9 ± 0.4	17.6 ± 0.3[d]	19.0 ± 0.9[c]	19.3 ± 0.4	18.5 ± 0.1	19.2 ± 0.2
18:2n-6	11.3 ± 0.2	13.8 ± 0.1[c]	16.7 ± 0.3[c,d]	8.5 ± 0.1	14.2 ± 0.1[c]	17.2 ± 0.3[c,d]
18:3n-3	1.3 ± 0.1	1.6 ± 0.1	1.7 ± 0.1[e]	1.1 ± 0.1	1.6 ± 0.1[e]	1.6 ± 0.1[e]
20:1n-9	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
20:2n-6	1.0 ± 0.1	1.4 ± 0.1	1.6 ± 0.1[c]	0.6 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
20:4n-6	2.0 ± 0.1	2.0 ± 0.1	1.7 ± 0.2	2.4 ± 0.1	2.4 ± 0.1	2.2 ± 0.1
20:5n-3	5.3 ± 0.1	6.0 ± 0.1[e]	4.9 ± 0.6	6.0 ± 0.1	5.7 ± 0.2	5.6 ± 0.1[d]
22:6n-3	29.1 ± 0.2	23.9 ± 0.3[c]	17.2 ± 2.2[c]	24.6 ± 0.4	20.5 ± 0.6[c]	15.9 ± 0.4[c]
Others[f]	3.3 ± 0.3	3.0 ± 0.1	3.0 ± 0.1	3.1 ± 0.2	2.9 ± 0.2	3.2 ± 0.3
ΣSFA	29.7 ± 1.3	28.8 ± 1.7	31.2 ± 1.5	32.3 ± 1.2	31.0 ± 0.9	31.5 ± 0.7
ΣMUFA	19.7 ± 1.0	21.9 ± 1.6	24.5 ± 2.0	23.8 ± 1.0	23.1 ± 1.3	24.3 ± 0.9
ΣPUFA	51.0 ± 2.3	49.6 ± 1.7	44.6 ± 2.0	43.9 ± 2.0	46.1 ± 2.1	44.3 ± 1.5
Σn-3	36.4 ± 1.0	32.2 ± 0.9	24.4 ± 0.5[c]	32.2 ± 0.5	28.4 ± 0.4[c]	23.8 ± 0.3[c,e]
Σn-6	14.6 ± 1.0	17.4 ± 0.2	20.2 ± 0.3[c]	11.7 ± 0.2	17.7 ± 0.5[c]	20.5 ± 0.7[c]
I	2.61 ± 0.05	2.42 ± 0.02	2.04 ± 0.1[c]	2.35 ± 0.05	2.23 ± 0.03	2.01 ± 0.02
I × 100/ΣSFA	8.89 ± 0.10	8.41 ± 0.10[c]	6.54 ± 0.02[c]	7.33 ± 0.10	7.05 ± 0.10	6.38 ± 0.10[c]
n-3/n-6	2.49 ± 0.02	1.85 ± 0.05[d]	1.21 ± 0.03[c,d]	2.75 ± 0.05	1.61 ± 0.05[c]	1.16 ± 0.07[c,g]

[a] Acronyms are listed in Materials and Methods.

[b] Numbers of pools analyzed. Values are means of n pools ± SE.

[c] $p \leq 0.01$, significance with WA; [d] $p \leq 0.01$, significance with SCA; [e] $p \leq 0.05$, significance with WA.

[f] See footnote of Table 1.

[g] $p \leq 0.05$, significance with SCA.

and in the species used. Roche and Pérès (27) reported a cold-promoted unsaturation increase of liver triglycerides in 12°C-acclimated sea bass. As the unsaturation increase was due to changes in different FA depending on the season, the authors concluded that cold acclimation processes were ruled by an internal clock not only that would be susceptive to the environmental temperature but also to the season. Moreover, at 12°C, sea bass were eating and thus, in our opinion, could adequately select dietary FA in response to thermal stress. The dietary intake of FA is crucial in marine teleosts because of the poor efficiency of PUFA biosynthesis (3,9,12,31–34).

These observations clearly strengthen our case to carry out experiments on cold-acclimation by following the seasonal cycle and to reproduce the natural winter conditions of reared sea bass in Northern Italy (11). The apparent lack of classical cold-adaptational mechanisms, *i.e.* a SFA decrease and a long-chain PUFA increase (1–5), suggests that such mechanisms do not represent the main strategy of sea bass to cold adaptation, and that other processes are likely to be involved.

Most cold acclimation studies have been carried out on winter-active freshwater teleosts such as the trout. These species exhibit far different features from the carnivorous and prevalently marine sea bass. In the literature on trout, feeding and normal activity were maintained during the whole experiment. However, below 10–12°C, sea bass strongly reduce food intake, as well as activity, and rest quite immobile at the bottom (12). Such a species-specific behavior may be related to a different, cold-directed metabolic modulation. The trout, not a strictly carnivorous species, shares with other freshwater teleosts the biosynthetic enzymes capable of forming long-chain n-3 and n-6 PUFA from the precursors 18:3n-3 and 18:2n-6 (3). Conversely, the sea bass is only able to synthesize long-chain PUFA to a limited extent as are most marine teleosts (3,9,12,31–34). These differences may explain the differential responses to low temperatures between the sea bass and freshwater teleosts.

Effect of cold acclimation on mitochondrial respiratory activities. Liver and heart mitochondrial oxidative activities show a clear thermoacclimation response (Table 5). However, the response to cold appears to depend on the tissue and the complexes involved.

State-3 and state-4 respiratory activities increase in liver mitochondria of LCA fish when glutamate is used as substrate, remain invariable with succinate, and decrease with ascorbate + TMPD. Thus RCR and ADP:O ratio values are generally constant.

Heart mitochondria exhibit a more homogenous behavior with all substrates; generally both state-3 and state-4 respiratory activities decrease in SCA and LCA groups. The ADP:O ratio is generally constant and RCR exhibits an increase with glutamate as a result of nonparallel variations undergone by states 3 and 4. Moreover the disappearance of coupling with ascorbate + TMPD during prolonged cold exposure may be related to the low RCR and its greater susceptibility to various perturbations (16). The overall decrease of mitochondrial respiratory activity during cold acclimation, excluding glutamate oxidation in liver mitochondria, strongly contrasts with literature reports. Acclimation indices (cold-acclimated/warm-acclimated) of enzymes involved in energy production are reported to be greater than 1 (2,30,35,36). Fish respiratory-chain enzymes are quoted as representative of positive compensation (2). The sea bass behavior may have an adaptive significance as it may reflect a cold-promoted reduction of all physiological activities and thus of energy requirements. This would be consistent with the less active succinate oxidation in slow fish with respect to fast

TABLE 5

Parameters of Liver and Heart Mitochondrial Respiration[a]

	Liver			Heart		
Substrate Parameter	WA n[b] = 6	SCA n = 2	LCA n = 4	WA n = 6	SCA n = 2	LCA n = 4
Glutamate						
State 3[c]	6.6 ± 1.0	6.4 ± 0.3	13.1 ± 0.6[d]	59.4 ± 6.9	58.4 ± 10.9	19.9 ± 7.9[d,e]
State 4[c]	2.6 ± 0.1	2.7 ± 0.1	3.4 ± 0.1[d]	4.5 ± 0.4	2.2 ± 0.2[f]	1.9 ± 0.1[d]
RCR	2.6 ± 0.4	2.9 ± 0.7	3.8 ± 0.3	14.3 ± 2.5	25.9 ± 2.6[f]	11.0 ± 4.0
ADP:O	2.1 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	2.6 ± 0.1	2.7 ± 0.1	2.8 ± 0.2
Succinate						
State 3	18.7 ± 1.7	19.4 ± 0.2	16.9 ± 0.9	36.8 ± 3.2	29.6 ± 0.9	25.0 ± 7.3
State 4	8.7 ± 0.3	8.3 ± 0.8	7.4 ± 0.6	12.2 ± 0.6	8.0 ± 0.6[d]	7.8 ± 1.5[f]
RCR	2.1 ± 0.2	2.4 ± 0.3	2.3 ± 0.0	3.0 ± 0.2	3.8 ± 0.2	2.6 ± 0.5
ADP:O	1.4 ± 0.0	1.5 ± 0.1	1.6 ± 0.2	1.3 ± 0.0	1.5 ± 0.0	1.2 ± 0.1
Ascorbate + TMPD						
State 3	148.4 ± 13.7	98.7 ± 18.5	81.9 ± 14.3[d]	286.1 ± 34.1	296.8 ± 15.6	200.1 ± 62.7[e]
State 4	134.3 ± 10.7	89.8 ± 17.0	n.d.[g]	247.6 ± 99.7	206.0 ± 10.9	n.d.
RCR	1.1 ± 0.0	1.1 ± 0.0	n.d.	1.2 ± 0.1	1.4 ± 0.0	n.d.
ADP:O	0.6 ± 0.1	0.7 ± 0.1	n.d.	0.6 ± 0.1	0.6 ± 0.0	n.d.

[a] Acronyms are listed in Materials and Methods.

[b] Number of pools analyzed. All values are means of n determinations ± SE.

[c] Respiratory activities are expressed as nmoles $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ at 20°C.

[d] $p \leq 0.01$, significance with WA; [e] $p \leq 0.05$, significance with SCA; [f] $p \leq 0.05$, significance with WA.

[g] Not detectable because all mitochondrial preparations were uncoupled.

swimmers (37). In nature the reduction of life processes during winter may represent a strategy adopted by sea bass to face unfavorable environmental conditions.

Results different from the commonly reported positive compensation in cold acclimation were found by Wodtke (38) in carp liver. He demonstrated that a transient increase in mitochondrial succinate oxidation rate, shown immediately (two days) after cold transfer, disappeared after prolonged (one month) exposure at 10°C.

The dependence on assay temperature of liver mitochondrial respiratory activity with ascorbate + TMPD as substrate (cytochrome oxidase) is shown as Arrhenius plot (Fig. 2). All plots are discontinuous with a break at about 20°C. The break is always clearly detectable as the ratio of the two activation energies below (Ea_2) and above (Ea_1) the break is approximately 2.0. Although various authors (14,39-43) have reported linear Arrhenius plots for fish membrane-bound enzymes, the occurrence of a break, as in the homeotherms (2), is widely documented (5,18,37,38,44,45). The temperature of discontinuity of the Arrhenius plot, in turn, is related to the temperature range of the phase transition of membrane lipids, and is generally influenced by the acclimation temperature, which is lowered in cold-acclimated fish as a result of an increase in PUFA (2,37,38,44). Thus, in the present experiments, the break constancy is consistent with the observed lack of substantial variations in the unsaturation parameters of the FA. The higher position of the WA plot with respect to positions of the SCA and LCA plots confirms the decrease in activity during cold-acclimation. Most of the decrease occurs during the first cold-adaptation period (SCA fish). Over the temperature range tested, the enzyme activities determined at high and low temperatures are never lower than those predicted on the basis of the linearity of both the upper and lower parts of the Arrhenius plots. The results suggest a similar thermosensitivity in differently acclimated sea bass (WA, SCA and LCA groups), at least in the temperature range considered. The temperature of discontinuity and both activation energies do not show any significant variations that can be attributed to cold acclimation.

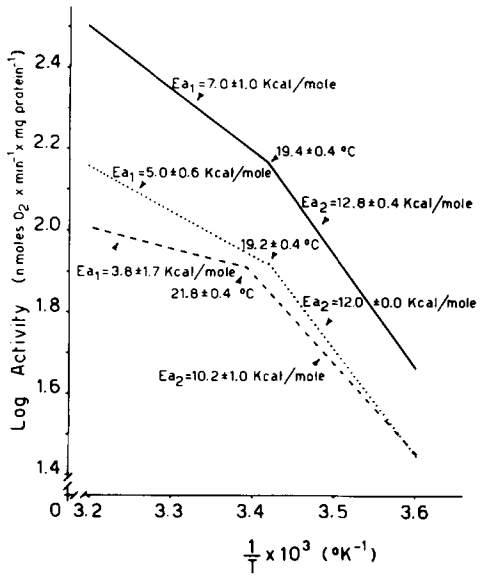

FIG. 2. Arrhenius plot of liver cytochrome oxidase system of WA ——, SCA ····· and LCA —— — fish in the temperature range 40-3°C. Ea_1, Ea_2, activation energies, expressed as Kcal/mole, above and below the temperature of discontinuity. The slopes were calculated from the average activation energies. The point of intersection corresponds to the average temperature of discontinuity (x-axis) and Log enzyme activities (y-axis) from two series of determinations of enzyme activity with no significant variations. In each series of determinations the lines were the best fit from least squares linear regression analysis.

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METHOD

Improved Resolution of ^{31}P Nuclear Magnetic Resonance Spectra of Phospholipids from Brain

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A method is described wherein the resolution of ^{31}P nuclear magnetic resonance spectra of the lipophilic fraction from a Bligh-Dyer extract of rat brain can be enhanced. The lipids are dispersed as micelles in aqueous solution with sodium deoxycholate, and spectral resolution is further optimized by adjusting the pH and by removing ions from the solution. The application of the method to the study of aging in rat brain serves as an example.
Lipids 27, 389-391 (1992).

Changes in phospholipid metabolism which occur in rat brains with aging have been demonstrated previously by ^{31}P nuclear magnetic resonance (NMR) (1,2). However, the studies were mainly concerned with the water-soluble metabolites, such as phosphocholine, glycerophosphocholine, phosphoethanolamine and glycerophosphoethanolamine. To obtain complementary data on more hydrophobic metabolites, including the phospholipids of neural membranes, is of considerable interest.

The Bligh-Dyer modified method has been shown to be most useful for extracting lipids (3,4). Although it gives a water-soluble fraction that produces sufficiently well-resolved ^{1}H and ^{31}P NMR spectra, the resolution achieved for the lipophilic fraction is usually not satisfactory. This makes qualitative and quantitative evaluation by NMR difficult.

In order to overcome these difficulties, a number of techniques have been proposed, including the formation of mixed micelles in aqueous medium by means of detergents (5-7), as well as the use of cesium-containing reagents (8).

In the present paper we report results obtained on rat brain extracts in a study on aging. The simple procedure we describe gives good spectral resolution of the phospholipid ^{31}P NMR signals obtained on the organic phase of the Bligh-Dyer extract.

MATERIALS AND METHODS

Six adult (6 months) and 12 aged (24 months) Fischer 344 male rats (Charles River, Calco, Italy) were sacrificed as previously described (9). The brains were separated from the cerebella and were crushed and pulverized at liquid N_2 temperature. The extraction was done with $CHCl_3$/CH_3OH/H_2O (2:2:1, v/v/v) following the technique by Bligh and Dyer (3) as modified by Miccheli *et al.* (4). The organic phase was separated and then washed with an equal volume of aqueous 100 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical Co., St. Louis, MO) at pH 7. The solution was dried under N_2 until the solvent was completely evaporated, and sodium deoxycholate (Sigma) was added to the lipid residue in a 3:1 (w/w) ratio. The sample obtained was then taken up in D_2O (99.95%; Farmitalia Carlo Erba, Milan, Italy) and an aliquot of a 100 mM Na_3PO_4 stock solution was added as internal reference. The pH was adjusted with 0.1 M NaOH to achieve the best resolution of spectral lines (about 11.4). The dispersion containing about 40 mM total phospholipids was sonicated in a bath sonicator at alternating 30 second intervals until an opalescent appearance was attained (5).

Proton decoupled ^{31}P NMR spectra were measured on a Varian XL-300 spectrometer (Varian Associates, Palo Alto, CA) operating at 121.4 MHz using an acquisition time of 1.5 seconds, a pulse length of 10 μs (45° pulse), and a pulse delay of 1.5 seconds. To ensure that complete relaxation had occurred, control spectra were measured with a pulse delay of 80 seconds; no differences in peak intensities were observed.

The chemical shifts of the phosphorus signals were measured relative to phosphonitrilic chloride (trimer) in benzene as an external reference. Values are reported in parts per million (ppm) relative to H_3PO_4, 85% (trimer, 20.8 ppm) (10). Assignments were made based on chemical shift values reported in literature and by comparison with pure standards.

Statistical analyses were by Student's *t*-test. The difference between two means was considered insignificant when $P > 0.05$.

RESULTS AND DISCUSSION

Figure 1a shows the ^{31}P NMR spectrum of the organic phase of the Bligh-Dyer extract from an adult rat brain redissolved in $CDCl_3$/CD_3OD (2:1, v/v). Figure 1b shows a spectrum of an analogous sample in aqueous dispersion after treatment with sodium deoxycholate. Seven major signals are present in the lower spectrum (Fig. 1b) which can be assigned to phosphatidic acid (PA), PO_4^{3-}, phosphatidylethanolamine (PtdEtn) + PtdEtn plasmalogen, phosphatidylserine (PtdSer), sphingomyelin (CerCho), phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCho) + PtdCho plasmalogen. The assignments and chemical shift values are listed in Table 1. All signals show a half-height bandwidth of less than 8 Hz.

In the $CDCl_3$/CD_3OD spectrum (Fig. 1a), the peak at 1.31 ppm corresponds to the overlapping signals of PtdEtn and PtdSer. However, these two signals are well resolved in the spectrum (Fig. 1b) of the deoxycholate

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Abbreviations: CerCho, sphingomyelin; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.

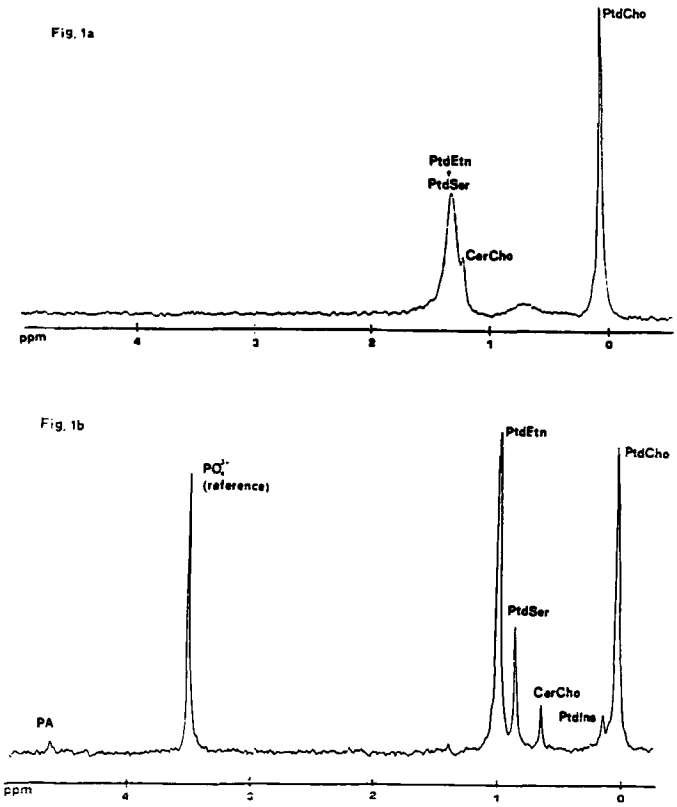

FIG. 1. a: ^{31}P NMR spectrum of the Bligh-Dyer organic phase from adult rat brain in $CDCl_3/CD_3OD$, 2:1 (v/v). b: ^{31}P NMR spectrum of the same sample in D_2O after treatment with EDTA and sodium deoxycholate followed by sonication (pH 11.4).

TABLE 1

^{31}P NMR Chemical Shifts of the Major Phospholipids from Rat Brain[a]

	Chemical shift (ppm)
PA	4.621
PO_4^{3-}	3.509
PtdEtn	0.989
PtdSer	0.855
CerCho	0.657
PtdIns	0.168
PtdCho	0.044

[a]ppm from H_3PO_4 (85%); for sample preparation, see Materials and Methods (data from Fig. 1b).

treated sample in water (0.989 and 0.855 ppm, respectively). In Figure 1a, the signal corresponding to CerCho is only a shoulder of the PtdEtn/PtdSer peak, while it is well-resolved at 0.657 ppm in the spectrum obtained after deoxycholate treatment. The signal corresponding to PtdIns is not visible in the $CDCl_3/CD_3OD$ spectrum, while it occurs as a low intensity peak at 0.168 ppm after treatment of the sample with detergent.

Meneses and Glonek (8) had previously analyzed (by NMR) phospholipid mixtures from animal and plant extracts by using another approach to minimize aggregation, which produced spectral half-height bandwidths of 1.8–3.2 Hz at a magnetic field strength of 11.75 T. Although this technique gave qualitative and quantitative data comparable to those obtained by chromatographic techniques, sample preparation is more complex. Use of detergents with an excess of EDTA to form micelles in aqueous medium was described by London and Feigenson (5). However, their spectra, measured at pH 8, did not show optimal resolution, particularly for PtdEtn, PtdSer and CerCho. Sappey Marinier *et al.* (6) employed a similar approach and evaluated the effect of pH and detergent/phospholipid ratio using Triton X-100 as detergent. The presence of metal cations appeared to be responsible for the wider bandwidths and for observed differences in chemical shift values reported by these authors.

The technique described here seems to provide an excellent alternative to previous methods. The absence of cations minimizes various ion effects on the polar phospholipid headgroups which contributes to a reduction in bandwidth of signals obtained on micelles formed by sonication in the presence of deoxycholate. The resolution thus attainable allows the qualitative and quantitative determination of the different phospholipid components.

Sotirhos *et al.* (11) had obtained ^{31}P spectra with a similar resolution on egg and liver extracts using hexane/propanol (3:2, v/v) as solvent. The Bligh-Dyer extraction, however, offers distinct advantages as it is quantitative, and eliminates lipid hydrolysis which can occur in the presence of perchloric acid (4). In addition, the Bligh-Dyer method gives a hydrophilic and a lipophilic phase from the same sample, each of which lend themselves to further study.

The possibilities offered by the present deoxycholate method allowed us to examine various cerebral extracts from adult (6 months) and aged (24 months) rats. The data obtained are reported in Table 2. The concentrations are expressed as percentages of total phosphorus content. The comparison between the phospholipid levels in the two populations shows no significant differences in PtdEtn, PtdSer, PtdIns and PtdCho, while a significant increase (+31.77%; $p < 0.005$) in CerCho levels is observed in the aged rats.

An increase in CerCho associated with an increase in PtdSer during the developmental process in rat brain was previously reported by Wells and Dittmer (12). This was assumed to be the result of an active myelination process.

TABLE 2

Phospholipid Composition of Bligh-Dyer Extract from Adult and Aged Rat Brains[a]

	Adult (n=6)		Aged (n=12)	
	Mean	SD	Mean	SD
PA	1.02	0.17	1.35	0.13
PtdEtn	43.92	2.09	43.08	1.67
PtdSer	11.68	0.98	11.92	1.03
CerCho[b]	2.99	0.54	3.94	0.66
PtdIns	2.78	1.11	3.24	0.79
PtdCho	34.76	2.65	34.01	1.37

[a]Values are expressed as percent of total phosphorus content.
[b]$P < 0.005$.

Variations in CerCho metabolism are known to occur in various pathological states, including cancer, atherosclerosis and Niemann-Pick disease (13).

Our present data do not allow us to clarify the causes of the observed phenomenon. Increased CerCho levels may be the result of decreased sphingomyelinase activity during aging, as it has been proposed for similar variations observed in aorta and in the lens of the eye (14,15).

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One-Step Synthesis of Radioactive Acyl-CoA and Acylcarnitines Using Rat Liver Mitochondrial Outer Membrane as Enzyme Source[1]

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Rat liver mitochondrial outer membrane enriched preparations have proven to be a convenient enzyme source for synthesizing coenzyme A (CoA) and carnitine esters of radioactive fatty acids. These membranes are simple to isolate and they retain acyl-CoA ligase and carnitine palmitoyltransferase activities well upon storage. Enzyme purification is not required. A novel aspect of the present procedure is that the same enzymatic incubation step allows both the acyl-CoA and the acylcarnitine esters to be obtained simultaneously when carnitine is present, but produces acyl-CoA ester only when carnitine is not included. Under the conditions described, the conversion of [1-^{14}C]octanoic acid to the respective esters was about 95%; the corresponding figure for [1-^{14}C]palmitic acids was over 70%. The procedure seems suitable for synthesizing the labeled CoA and carnitine esters from a variety of radioactive fatty acids.

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Metabolic fatty acid studies frequently require the use of radioactive coenzyme A (CoA) and carnitine esters. However, these derivatives are not readily available even for some of the more common fatty acids. Also, several chemical methods that have been described do not give high yields when working with submicromolar quantities (see refs. 2 and 3). While procedures for synthesizing acyl-CoA esters of radioactive fatty acids have been described (4–6), micromethods for synthesizing labeled acyl carnitines in acceptable radioisotopic yields are not available.

We describe here a novel enzymatic procedure using an outer membrane enriched preparation of rat liver mitochondria as the source of the fatty acyl-CoA ligase and carnitine acyltransferase activities. The procedure allows us to obtain the fatty acyl esters of carnitine and CoA by the same incubation. The system yields acyl-CoA ester alone when carnitine is not included. The procedure is relatively simple, does not require purification of enzymes, and gives radioisotopic yields of 70–95% with [1-^{14}C]-palmitic acid and [1-^{14}C]octanoic acid, respectively, as substrates.

EXPERIMENTAL PROCEDURES

Isolation of an outer membrane enriched preparation from mitochondria. Rat liver mitochondria and outer membrane enriched preparations are isolated by previously described procedures, except for minor modifications (7–10). The isolation procedures are carried out at 0–4°C. Freshly removed rat livers (*ca.* 60 g) are washed, minced, and a 15% (w/v) homogenate is prepared in 210 mM mannitol, 70 mM sucrose, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4). The homogenate is centrifuged for 10 min at 500 × *g* and the resulting supernatant is spun at 1500 × *g* for 5 min, then at 8,000 × *g* for 10 min. The supernatant, along with the lightly packed layer on the surface of the pellet, is aspirated off and the pellet is rinsed twice with a small volume of the homogenizing medium to further remove the lightly packed material. The pellet is suspended in fresh homogenizing medium and then centrifuged for 3 min at 500 × *g* to sediment any remaining blood cells, etc. The supernatant is then spun at 8,000 × *g* for 10 min. The resulting pellet is suspended in fresh homogenizing medium, recentrifuged for 10 min at 8,000 × *g*, then rinsed as above to remove the lightly packed material. The rinsed pellets are finally suspended in 10 mL of the homogenizing medium yielding a mitochondrial fraction that contains 750–950 mg protein.

To obtain outer membrane enriched vesicles (OMV) by the swelling/shrinking procedure, the above mitochondrial suspension is diluted to 300 mL with 20 mM potassium phosphate, pH 7.2, containing 0.02% bovine serum albumin, and left at 0°C for 20 min. Then 37 mL of 15 mM ATP and 37 mL of 15 mM $MgCl_2$ are added. After 4 min at 0°C, the suspension is spun at 35,000 × *g* for 20 min, and the pellets are suspended in about 100 mL of 20 mM potassium phosphate (pH 7.2) containing 0.02% bovine serum albumin, 1.5 mM ATP, and 1.5 mM $MgCl_2$. The suspension is spun at 4,000 × *g* for 10 min, and the pellet is discarded. The supernatant is recentrifuged at 150,000 × *g* for 60 min. The resulting pellets are suspended in 3.8 mL of 20 mM potassium phosphate (pH 7.2), and 0.6-mL aliquots are layered separately, in six centrifuge tubes (SW 60Ti rotor) on top of a sucrose gradient; the sucrose gradient consists, from bottom to top, of 1 mL of 51.3% sucrose in 20 mM potassium phosphate, 1 mL of 37.7% sucrose in 20 mM potassium phosphate, and 1 mL of 25.3% sucrose in 20 mM potassium phosphate. The tubes are centrifuged at 115,000 × *g* for 60 min. The first top band containing mostly outer membrane fragments with fatty droplets is aspirated off. The second band from top, at the interphase of 25.2% and 37.7% sucrose, is collected, diluted 1:4 with chilled 20 mM potassium phosphate (pH 7.4) having protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 25 μg/mL leupeptin, 20 μg/mL pepstatin) and spun again at 150,000 × *g* for 60 min. The pellet obtained is suspended, by gentle vortexing, in 1 mL of 250 mM sucrose and 5 mM potassium phosphate (pH 7.2) containing protease inhibitors as above, to obtain an outer membrane enriched preparation (OMV) containing 10–15 mg protein.

[1] A preliminary account of this work has been published (ref. 1).

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Abbreviations: CoA, coenzyme A; CPT_o, outer carnitine palmitoyltransferase; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; OMV, outer membrane enriched vesicles.

The OMV thus prepared show a parallel enrichment of the specific activities of the outer membrane marker, monoamine oxidase, and of the malonyl-CoA sensitive carnitine palmitoyltransferase which is 12–20-fold enriched over the activity in the mitochondrial preparation (9). Aliquots of OMV are stored at −80°C, and can be used for acyl-CoA and acylcarnitine syntheses for at least three months (the longest period tested). The palmitoyl-CoA synthetase activity of these OMV is about 625 nmol/min/mg protein (9). The specific activity of outer carnitine palmitoyltransferase (CPT_o) with palmitoyl-CoA as substrate varies from 40 to 70 nmol/min/mg under the assay conditions described previously (9) at 2 mM carnitine. The CPT_o specific activity with octanoyl-CoA as substrate is 150–230 nmol/min/mg when assay conditions are as described by Murthy *et al.* (11), except that the concentration of potassium phosphate is 100 mM and that of carnitine is 5 mM. No noticeable change in activities is found following storage of OMV preparations at −80°C for 3 months.

Synthesis of palmitoyl-CoA and palmitoylcarnitine. The reaction system contains, in 1 mL, 250 mM potassium phosphate (pH 7.4), 1.9 mM CoA, 5 mM ATP, 1 mM L-carnitine, 10 mM $MgCl_2$, 1 mg bovine serum albumin, 185 nmole [1-^{14}C]palmitate (10 μCi), and OMV corresponding to 50 μg of protein. Incubations are carried out for 90 min at 37°C with shaking. Reactions are stopped by the addition of 50 μL of 5 M HCl, and chilling. Unused palmitic acid is removed by extracting four times with 3-mL portions of diethyl ether. The aqueous phase is then extracted three times with 3-mL portions of water-saturated butanol. The combined butanol phases are washed twice with 2 mL of butanol-saturated water and are then lyophilized. The residue is taken up in 2 mL of 80% (v/v) methanol, its pH is brought to 5–6 with 1 M KOH, and then the solution is passed through a DEAE Sephacel column (acetate form, 5 mm × 50 mm, equilibrated with 80% methanol). A further 6 mL of 80% methanol is then passed through the column to complete palmitoylcarnitine elution. The palmitoyl-CoA is then eluted from the DEAE Sephacel column with 9 mL of 80% methanol containing 10 mM acetic acid and 0.5 M ammonium acetate. The solvent is evaporated under a stream of nitrogen, and the material is lyophilized. The radioactive palmitoyl-CoA thus obtained, while radioisotopically pure, shows some carryover of CoA as a contaminant. A CoA-free product is obtained, if desired, by using a further high-pressure liquid chromatographic step (HPLC) as described later in Results and Figure 2. The palmitoyl-CoA peak is collected and the acetonitrile is evaporated under a stream of nitrogen. The aqueous residue is lyophilized, and the dried material is extracted with methanol/butanol (1:1, v/v), leaving behind potassium phosphate.

Synthesis of octanoyl-CoA and octanoylcarnitine. The reaction system contains, in 1 mL, 250 mM potassium phosphate (pH 7.4), 1.9 mM CoA, 5 mM ATP, 1 mM L-carnitine, 10 mM $MgCl_2$, 170 nmole [1-^{14}C]octanoic acid (10 μCi), and 50 μg OMV protein. Incubations are for 90 min at 37°C with shaking. Reactions are stopped by the addition of 50 μL of 5 M HCl, and chilling. Unused octanoic acid is removed by extracting four times with 3-mL portions of diethyl ether. The pH of the aqueous phase is brought to 5–6 by addition of 1 M KOH, and then the solution is passed through a DEAE Sephacel column (acetate form, 5 mm × 50 mm, equilibrated with 80% methanol). Six mL of 80% methanol is further passed through the column to completely elute octanoylcarnitine and carnitine together. Nine mL of 80% methanol containing 10 mM acetic acid and 0.5 M ammonium acetate is then passed through the column to elute octanoyl-CoA and CoA together.

The octanoylcarnitine is separated from free carnitine by blowing off the methanol under nitrogen, dissolving the residue in 1 mL of water, and extracting the aqueous phase three times each with 3 mL of water-saturated butanol. The combined butanol phases are washed twice with 2 mL of butanol-saturated water and are then lyophilized to obtain octanoylcarnitine.

For isolating octanoyl-CoA, the ammonium acetate eluent is lyophilized, the residue is taken up in 1.5 mL of water, and pH is adjusted to 5–6. If a CoA-free product is desired, aliquots are further purified by HPLC (see below). Otherwise the solution is applied onto a 3-mL, open, pre-packed C_{18} reverse phase column (Supelclean of Supelco, Oakville, Ontario, Canada; washed with water, methanol, then water). The eluent is passed through the column three more times, then the column is washed with 2 mL of acetonitrile/50 mM potassium phosphate (5:95, v/v; pH 5.6). This elutes most of the CoA. Octanoyl-CoA is next eluted with 5–6 mL of acetonitrile/50 mM potassium phosphate (60:40, v/v; pH 5.6). The eluent contains radioisotopically pure octanoyl-CoA (see Results and Fig. 2), but a variable CoA contamination is frequently found upon analyzing the product by reversed phase HPLC. When a CoA-free product is desired, it is obtained by omitting the above open column step. The material is instead purified by reversed phase HPLC (see Fig. 2 for details). The octanoyl-CoA peak is collected, and the eluent is removed by a stream of nitrogen. The residual aqueous phase is lyophilized and the dried residue is extracted with methanol/butanol (1:1, v/v) to eliminate potassium phosphate.

RESULTS AND DISCUSSION

Table 1 shows that with [1-^{14}C]octanoic acid and [1-^{14}C]-palmitic acid, the incubation produced the corresponding esters of CoA or of CoA and carnitine in isotopic yields of about 95 and 74%, respectively. These yields were not noticeably affected by variations in incubaton time (from 30 to 180 min) or by variations in the quantity of OMV protein used (from 5 to 200 μg). Yields started to decline only when more than 300 μg of protein was present; yields were 68% with 1 mg OMV protein. Use of a high salt concentration, *i.e.*, 250 mM potassium phosphate, at the incubation step made it possible to lower the quantity of OMV protein to 5 μg and still obtain optimal yields of the acyl esters (Table 1). This is likely the result of an activating/stabilizing effect of the salt on the outer carnitine palmitoyltransferase (12) and acyl-CoA ligase activities (13). With freshly dissolved samples of CoA, inclusion of 5 mM dithiothreitol did not change product yields.

Table 1 shows also that comparable yields of the CoA esters alone, of about 73 and 92% with palmitic and octanoic acids, respectively, are obtained when carnitine is not added. When carnitine is included, the final reaction mixture contains the acyl-CoA and acylcarnitine products;

TABLE 1

Conversion of Palmitic and Octanoic Acids to their CoA and Carnitine Esters as Effected by the Quantity of OMV Protein and Incubation Time[a]

[1-^{14}C]Fatty acid	OMV protein (μg)	Incubation time (min)	Carnitine	% Conversion to	
				Acyl-CoA ester	Acyl-CoA + acylcarnitine
Palmitic acid[b]	60	120	+		73-79
Palmitic acid	60	120	−	71-75	—
Octanoic acid[c]	1000	90	+	—	66-70
Octanoic acid	400	120	+	—	85-90
Octanoic acid	25-200	90	+	—	95 ± 2.1 (n=7)
Octanoic acid[b]	5-30	90	+	—	94 ± 7.1 (n=6)
Octanoic acid	30-60	90	−	94-97	—
Octanoic acid	300	15	+	—	65
Octanoic acid	300	30-180[d]	+	—	87-95

[a]Mean ± SD as shown.

[b]With 250 mM potassium phosphate (pH 7.4).

[c]With 100 mM potassium phosphate (pH 7.4).

[d]Six time points in between.

in several mixtures that were analyzed, the two acyl esters were found present in about equal proportions with both [1-^{14}C]palmitic acid and [1-^{14}C]octanoic acid.

Figure 1 shows the characterization of the synthesized [1-^{14}C]palmitoylcarnitine and [1-^{14}C]octanoylcarnitine as *p*-bromophenacyl derivatives by HPLC. In both cases, a single radioactivity peak emerged in the area expected, indicating that the acylcarnitines synthesized were radioisotopically pure. Similarly, HPLC of the [1-^{14}C]-palmitoyl-CoA and [1-^{14}C]octanoyl-CoA preparations (see Fig. 2 for details) showed that each of these products emerged at the expected retention time with 91-96% recovery of the radioactivity applied. No other radioactivity peaks were detected, confirming the radiopurity of the acyl-CoA products synthesized.

For the synthesis of acylcarnitines and acyl-CoA esters, we found the OMV of rat liver mitochondria to be a convenient source of the required enzyme activities. Firstly, the OMV are relatively simple to isolate and their use obviates the need for purifying the enzymes, and particularly the carnitine palmitoyltransferase. Secondly, OMV show high specific activities of the acyl-CoA ligase and the outer carnitine palmitoyltransferase (CPT_o) (see Methods and ref. 9), so that microgram quantities of OMV suffice for preparing microCurie quantities of the products. Thirdly, OMV acyl-CoA ligase and CPT_o activities are stable during storage as long as repeated freeze-thawing is avoided. No noticeable change in enzyme activities was observed following three months of storage of the OMV in the frozen state (−80°C) or as lyophilized material (the latter was reconstituted with water before use). The radioisotopic yields of the acyl-CoA and acylcarnitine esters obtained with frozen or lyophilized OMV preparations were similar to those obtained with freshly isolated OMV (74 ± 4% with [1-^{14}C]palmitic acid and 95 ± 6 [n = 8] with [1-^{14}C]octanoic acid). Fourthly, the OMV preparations showed little acyl-CoA hydrolase activity. Fifthly, use of OMV allows the acyl-CoA and acylcarnitine both to be synthesized simultaneously.

The details of the incubation and product isolation conditions described here are those found optimal for obtaining CoA and carnitine esters of palmitic and octanoic acids. The same procedure should, however, allow the CoA and carnitine esters of other fatty acids to be obtained in a like manner inasmuch as the acyl-CoA ligase and CPT_o of OMV show broad substrate specificity and the isolation steps also should be applicable for isolating the corresponding esters of other fatty acids. The methods should be of particular interest to those involved in studies on fatty acid oxidation. Several metabolic disorders related to impaired fatty acid oxidation recently have been recognized (17), the investigation of which frequently requires CoA and carnitine esters of different fatty acids.

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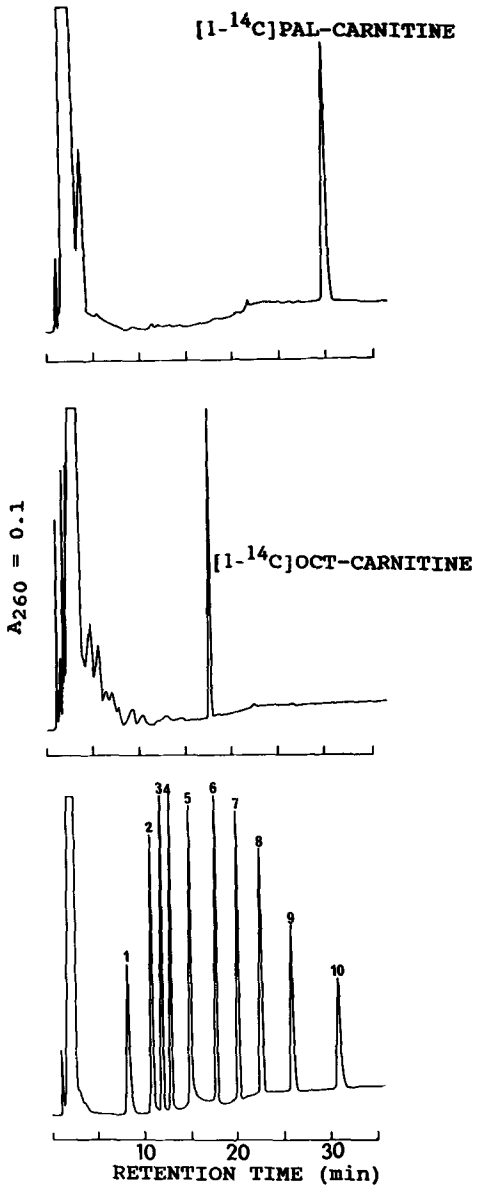

FIG. 1. Characterization of synthesized acylcarnitines as *p*-bromophenacyl derivatives by reversed phase HPLC. The *p*-bromophenacyl derivatives of the carnitines were prepared as in (14) and were resolved as in (15), except for minor modifications. A ternary gradient of acetonitrile (A), water (B) and 0.15 M triethylamine phosphate (pH 5.6) (C) was used as follows: Isocratic 70% (v/v) A, 28% B, 2% C, 6 min; linear gradient 70–80% A, 28–18% B, 2% C, 4 min; linear gradient 80–90% A, 2–10% C, 15 min; linear gradient 90–93% A, 10–7% C, 10 min; linear gradient 93–70% A, 0–28% B, 7–2% C, 5 min. The flow rate was 1.5 mL/min. A Varian 9010 solvent delivery system with a Supelcosil LC-8 (5 micron) column (25.0 cm × 4.6 mm) was used for HPLC. The top two panels show results obtained with the [1-^{14}C]palmitoylcarnitine and [1-^{14}C]octanoylcarnitine synthesized. The bottom most panel shows results obtained with a standard mixture of acylcarnitines that contained: 1, carnitine; 2, acetylcarnitine; 3, propionylcarnitine; 4, butyrylcarnitine; 5, hexanoylcarnitine; 6, octanoylcarnitine; 7, decanoylcarnitine; 8, dodecanoylcarnitine; 9, tetradecanoylcarnitine; and 10, hexadecanoylcarnitine.

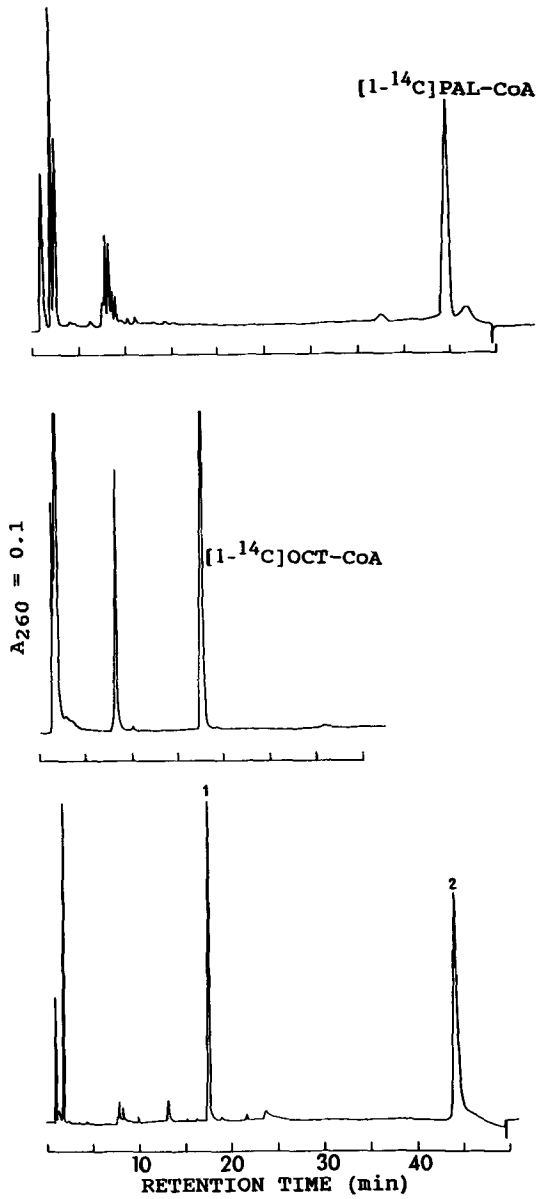

FIG. 2. Purification and characterization of synthesized acyl-CoA esters by reversed phase HPLC. The HPLC system is as in Figure 1, but an LC_{18} column was used. Acyl-CoA esters were resolved (16) by the following gradient of acetonitrile in 50 mM KH_2PO_4 (pH 5.3): Isocratic 5% (v/v), 5 min; isocratic 10%, 0.1 min; linear gradient 10–30%, 9.9 min; linear gradient 30–50%, 30 min; linear gradient 50–5%, 5 min. The flow rate was 2.0 mL/min. The top two panels show the results obtained with the synthesized [1-^{14}C]palmitoyl-CoA and [1-^{14}C]octanoyl-CoA, while the bottom most panel shows results with a standard mixture of octanoyl-CoA (peak 1) and palmitoyl-CoA (peak 2).

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COMMUNICATION

High-Performance Liquid Chromatographic Separation of Enantiomeric Benzyl Glycerides[1]

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The enantiomeric resolution of a series of 1,2-diacyl- and 1,2-mixed acid-diacyl-3-benzyl-*rac*-glycerols was investigated by high-performance liquid chromatography (HPLC). Of the racemic benzylglycerols studied, the 1-*O*-benzyl-2,3-*O*-iso-propylidene-*rac*-glycerol, 1-acyl-3-benzyl-*rac*-glycerol and 2-acyl-3-benzyl-*rac*-glycerol structures could be resolved into their enantiomers. The latter were resolved on a silica (10 μm) column coated with cellulose tribenzoate by isocratic elution with hexane/isopropanol mixtures as mobile phase. The effects of para substitution of the benzyl moiety on the resolution of the acylbenzylglycerols by this HPLC method were evaluated.
***Lipids* 27, 396–399 (1992).**

1,2-Diacyl-*sn*-glycerols are activators of protein kinase C (PKC), an enzyme which plays an important role in signal transduction (1–5). To investigate the structural requirement of diacylglycerols as protein kinase C activators, enantiomeric 1,2-diacyl-*sn*-glycerols have been synthesized (4,5). Typical synthetic methods for the synthesis of enantiomeric 1,2-diacyl-*sn*-glycerols require optically active precursors, such as D-mannitol (6,7). The procedures typically involve a number of synthetic steps, produce relatively low yields, and cause racemization during synthesis.

A variety of chiral stationary phase (CSP) high-performance liquid chromatography (HPLC) columns have been developed recently and have been used to separate chiral compounds into their optical isomers (8). CSP HPLC columns also have been used on a preparative scale to isolate pure enantiomers (9). Thus, it may be easier to separate by HPLC selected racemic intermediates into their enantiomers, rather than to synthesize enantiomers from optically active compounds as starting materials. Using this approach, we recently reported the separation of enantiomeric alkyl glycerol ethers on a cellulose tribenzoate CSP HPLC column (10).

In the present study, we investigated the enantiomeric separation of several synthetic intermediates that are encountered in the synthesis of 1,2-diacylglycerols. We investigated the separation of 1,2-monoacid-diacylglycerols as well as of 1,2-mixed acid-diacylglycerols because different synthetic routes are required for their preparation.

[1]Presented in part at the AOCS 81st Annual Meeting, Baltimore, Maryland, April 1990. Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Abbreviations: α, separation factor; CSP, chiral stationary phase; HPLC, high-performance liquid chromatography; IR, infrared; K', capacity factor; NMR, nuclear magnetic resonance; PKC, protein kinase C; R_s, resolution.

MATERIALS AND METHODS

Materials. Oleic acid (99%, Extra Oleic 99) was obtained from Nippon Oil & Fats Co. (Tokyo, Japan). All other reagents used for the syntheses were obtained from Aldrich Chemical Co. (Milwaukee, WI). *n*-Hexane and isopropanol used for HPLC were obtained from American Burdick & Jackson (Muskegon, MI).

Analytical system. The HPLC system used in this study was a Beckman Model 110A instrument (Beckman Instruments, San Ramon, CA) which was connected to an ultraviolet detector (Spectroflow 773, Kratos Instruments, Ramsay, NJ) and an HP 3396A integrator (Hewlett-Packard, Avondale, PA); a Waters Differential Refractometer Model R 401 detector (Waters Associates, Milford, MA) was used as required. The analytical HPLC column used was a Microsorb Si, 4.6 mm i.d. × 25 cm, 5 μm silica column (Rainin Instrument Co., Woburn, MA). The preparative silica column was a Dynamax Macro Si, 10 mm i.d. × 25 cm, 8 μm (Rainin Instrument Co.). The CSP HPLC column was a Bakerbond Chiralcel OB, 4.6 mm i.d. × 25 cm stainless steel column prepacked with 10 μm silica coated with cellulose tribenzoate (J.T. Baker, Phillipsburg, NJ). Columns were eluted isocratically with n-hexane/isopropanol (varying in composition from 96:4 to 99:1, v/v). In the case of the analytical column and the CSP column, sample concentrations were 1% (w/v) in mobile phase as solvent; injection volumes were 20 μL, and the flow rate was 0.5 or 1 mL/min. For preparative separations, sample concentrations were 50% (w/v), injection volumes were 100 μL, and the flow rate was 5 mL/min.

Syntheses. As shown in Figure 1, 1-*O*-benzyl-2,3-*O*-isopropylidene-*rac*-glycerol **2a** was synthesized by benzylation of racemic glycerol acetonide **1** with benzyl chloride in the presence of aq. NaOH and tetrabutylammonium bromide (11). 1-*O*-(4-Substituted-benzyl)-2,3-*O*-isopropylidene-*rac*-glycerols **2b–d** were obtained in a similar manner.

As illustrated in Figure 2, 1-benzyloxy- and 1-(4-substituted-benzyloxy)-2,3-epoxy-propanes **5a–c** were obtained by benzylation of epichlorohydrin **4** with benzyl or 4-substituted benzyl alcohols in the presence of aq. NaOH and tetrabutylammonium bromide. 1-*O*-Benzyl- and 1-*O*-(4-substituted-benzyl)-3-acyl-*rac*-glycerols **6a–c** were obtained by acylation of **5a–c** with fatty acid in the presence of tetraethylammonium bromide (12). Their 2-acyl-isomers **7a–c**, formed as co-products in the latter reaction, were separated and purified by preparative HPLC on the silica column.

All compounds were identified by infrared (IR) and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and purity confirmed by analytical HPLC (Table 1).

RESULTS AND DISCUSSION

Enantiomeric HPLC separation of synthetic intermediates for enantiomeric 1,2-monoacid-diacyl-glycerol. We previously reported that racemic glycerol acetonide **1** (Fig.

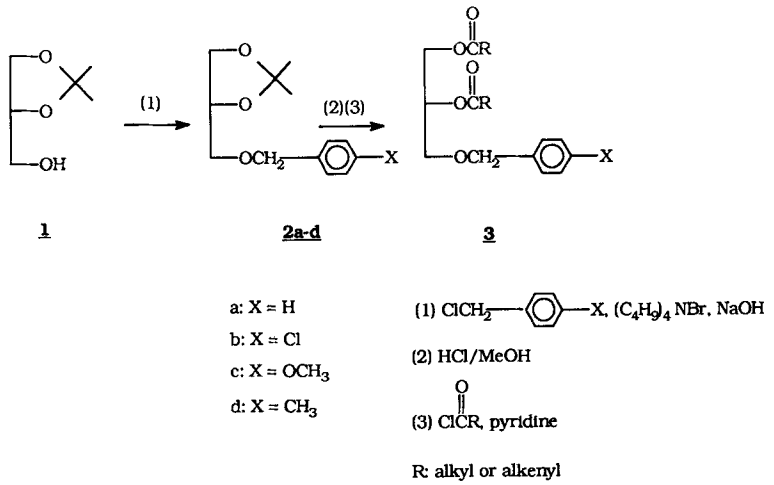

FIG. 1. Synthesis of racemic 1,2-diacylglycerols.

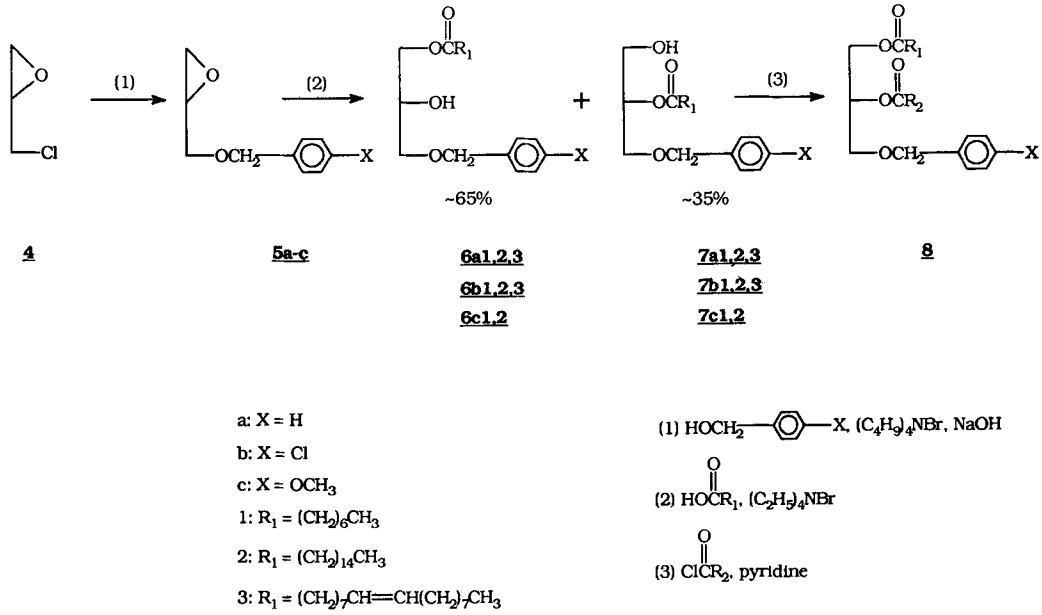

FIG. 2. Synthesis of racemic 1,2-mixed-acid-diacylglycerols.

1) and its 1-*O*-benzyl derivative **2a** (Fig. 1) can be separated into their enantiomers using a cellulose tribenzoate CSP HPLC column (abbreviated as "OB" below) (10). However, the enantiomers of glycerol acetonide may not be suitable synthetic intermediates for preparative isolation because they tend to racemize, and compound **2a** does not completely resolve into its enantiomer, which is required for preparative purposes (10). In an attempt to improve the enantiomeric resolution of **2a**, we prepared 4-substituted-benzyl analogs **2b–d** (Fig. 1) and examined their enantiomeric separation using the CSP HPLC column. Table 2 gives the chromatographic data obtained in the present study on the resolution of 1-*O*-benzyl and 1-*O*-(4-substituted-benzyl)-*rac*-glycerols. The separation factor (α) and resolution (R_s) values observed for **2a–d** were in the following order: compound **2c>2b>2d>2a**, which corresponds to a para substituent order of OCH_3>Cl>CH_3>H. Interaction between the phenyl moieties of the sample compound and the chiral stationary phase have been often cited in describing the mechanism of chiral recognition. It has been reported that electron-donating substituents on the phenyl moieties of the cellulose tribenzoate CSP (13) or the analyte compounds (14) improve the effective chiral recognition of the CSP. However, from our results for compound 2 (Fig. 1), it appears that the stronger the electron-donating substituent (*e.g.* OCH_3) or the electron-withdrawing (*e.g.* Cl) substituent effect on the phenyl moiety, the better an enantiomeric resolution is achieved.

We also examined the enantiomeric separation of several racemic 1,2-monoacid-diacyl-3-benzyl- or (4-substituted-benzyl-glycerols **3** (Fig. 1) on the CSP column; however, none of these intermediates could be separated into enantiomers.

TABLE 1

HPLC Separation of Various Substituted Benzyl-*rac*-glycerols[a]

Compound[b]	Retention time (min)	Chromatographic purity (%)	Mobile phase composition[c]
2a	4.5	94	98:2
2b	4.9	86	98:2
2c	5.4	87	98:2
2d	5.1	95	98:2
6a,1	11.1	88	98:2
6b,1	7.5	100	96:4
6c,1	8.3	100	96:4
6a,2	9.7	100	98:2
6b,2	11.9	95	98:2
6a,3	9.0	92	98:2
6b,3	8.4	97	97:3
71,1	17.9	100	98:2
7b,1	10.0	100	96:4
7c,1	11.1	100	96:4
7a,2	14.4	100	98:2
7b,2	18.1	89	98:2
7a,3	13.6	81	98:2
7b,3	12.0	88	97:3

[a]Separations were obtained using a microsorb Si column (25 × 0.46 cm) at a flow rate of 1 mL/min.

[b]Structure of compounds is given in Figures 1 and 2.

[c]Mobile phase, *n*-hexane/isopropanol (v/v).

TABLE 2

Enantiomeric Separation of 1-Benzylglycerols 2a–d

Compound	Capacity factor K′	Separation factor α	Resolution R_s
2a	3.31	1.19	0.70
2b	3.19	1.50	1.23
2c	11.08	1.54	1.46
2d	3.36	1.50	0.82

Because a desirable base-line separation (R_s>1), a criterion that must be met to resolve enantiomers, was obtained with **2b** and **2c**, we considered these compounds useful as synthetic intermediates for preparing diacylglycerols in which both acyl residues are the same (Fig. 1).

Enantiomeric HPLC separation of synthetic intermediates for the preparation of enantiomeric 1,2-mixed acid-diacyl glycerols. The synthetic scheme used for preparing racemic 1,2-mixed acid-diacylglycerols is shown in Figure 2 (11,12). Compounds **6a–6c** are candidates for separation into their enantiomers because they contain the requisite functional groups, namely an ester, a hydroxy group and an aromatic ring. We have previously shown that these structural features enhance the enantiomeric separation on the CSP column (10). In the present study, we also prepared several 1-*O*-benzyl- and 1-*O*-(4-substituted-benzyl)-3-acyl-glycerols **6a–c,1-3** to examine their enantiomeric separation on the CSP column. The 2-acyl isomers **7a–c,1-3**, which are formed as co-products, were purified and isolated by preparative HPLC and their enantiomeric separation was also investigated.

Table 3 gives the chromatographic data for the racemic 1-acyl and 2-acyl-3-benzyl-glycerols prepared in this study. Compounds **6a–c,1-3** were separated only partially into their enantiomers or were not separated at all on the CSP column. On the other hand, the corresponding 2-acyl isomers **7a–c,1-3** were resolved into their enantiomers with a good separation factor (α) and resolution (R_s) and with most giving a base-line separation (R_s>1). These results demonstrate that an ester group at the 2-position of the glycerol backbone contributes more substantially to the enantiomeric separation than does an ester group at the 3-position of the glycerol backbone. This is in agreement with our previous study where we reported that 1-*O*-hexadecyl-2-*O*-benzyl-*rac*-glycerol was successfully separated into its enantiomers by the CSP column, whereas the corresponding 3-benzyl isomer was not resolved at all (10). From our previous results and based on the present data, it is concluded that a selected functional group, *e.g.*, the ester or benzyl group, needs to be attached at the 2-position of the glycerol backbone to accomplish a successful enantiomeric separation on a CSP column.

The effect of the substituents on the phenyl moiety on enantiomeric separation was less clear because, in the case of the octanoyl and oleoyl glycerols **7a1** and **7a3**, the nonsubstituted benzyl derivatives showed better enantiomeric separation than the substituted benzyls **7b1** and

TABLE 3

Enantiomeric Separation of 1(2)-Acyl-3-benzylglycerols 6 and 7

	rac-Glycerol position			Enantiomeric separation		
	1[a]	2	3	K′	α	R_s
6a,1	phenyl		octanoyl	10.7	1.19	0.83
6b,1	4-chlorophenyl		octanoyl	12.7	1.22	0.81
6c,1	4-methoxyphenyl		octanoyl	28.3	1.10	0.4
6a,2	phenyl		palmitoyl	1.4	1.0	0.4
6b,2	4-chlorophenyl		palmitoyl	6.2	1.15	0.54
6a,3	phenyl		oleoyl	4.1	1.15	0.4
6b,3	4-chlorophenyl		oleoyl	5.0	1.0	0.4
71,1	phenyl	octanoyl		10.3	1.53	1.26
7b,1	4-chlorophenyl	octanoyl		13.9	1.18	0.81
7c,1	4-methoxyphenyl	octanoyl		26.4	1.33	1.04
7a,2	phenyl	palmitoyl		2.8	1.0	0.4
7b,2	4-chlorophenyl	palmitoyl		7.2	1.34	1.13
71,3	phenyl	oleoyl		4.8	1.60	1.40
7b,3	4-chlorophenyl	oleoyl		6.3	1.82	1.17

[a]The phenyl moiety referred to is that of the benzyl group located at position-1 of glycerol (see Fig. 2).

7b3. We also attempted to separate several racemic 1,2-mixed acid-diacyl-3-benzyl- or (4-substituted-benzyl)-glycerols 8 using the CSP column. However, enantiomeric separation was not achieved.

Based on the present data we conclude that 2-acyl-3-benzyl-glycerols, such as compounds **7a–c** (Fig. 2, Table 3), have the best potential as intermediates for the preparative enantiomeric separation of the racemic compounds. Because the 2-acyl-3-benzyl-glycerols are co-products of the synthetic pathway we have used (Fig. 2), alternate synthetic routes by which the 2-acyl isomers are obtained as major products are under investigation.

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REVIEW

Dietary Ether Lipid Incorporation into Tissue Plasmalogens of Humans and Rodents

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Chronic feeding of 1-*O*-octadecyl-*sn*-glycerol (batyl alcohol) to patients suffering from congenital deficiency in tissue ether glycerolipids showed an increase in the plasmalogens content of their erythrocytes. However, nothing is known about the ether lipid content of other tissues in these patients. Feeding 1-*O*-heptadecyl-*sn*-glycerol to young rats showed that this uncommon ether lipid was incorporated to a high extent into the plasmalogens of all tissues except brain. Comparative studies with other precursors, such as 3-*O*-heptadecyl-*sn*-glycerol, heptadecanol and heptadecanoic acid, indicated a stereospecific incorporation of the dietary 1-*O*-alkyl-*sn*-glycerols into tissue plasmalogens without cleavage of the ether bond. Dietary ether lipids were also shown to be transferred from mothers to suckling rats, but not from pregnant rats to fetuses. The implication of these results to possible dietary ether lipid therapy for patients suffering from peroxisomal disorders is discussed.
Lipids 27, 401–405 (1992).

In mammals, 1-*O*-alkyl-*sn*-glycerols derived from dietary ether glycerolipids are absorbed without cleavage of the ether bond (1). This was first shown by Bergström and Blomstrand (2) who fed radioactive 1-*O*-[1'-^{14}C]hexadecyl-*rac*-glycerol (chimyl alcohol) to rats and showed that it was completely absorbed by the intestine. About half of the radioactive lipids recovered from the lymphatic fluid were shown to be either chimyl alcohol or its esterified derivatives, and the rest of the radioactivity was recovered in palmitic acid. Using human subjects, a similar finding was reported by Blomstrand and Ahrens (3). Apparently after absorption, all of the unnatural isomer (*sn*-3-alkyl) and part of the natural (*sn*-1-alkyl) isomer were oxidized in the intestinal mucosal cells to form palmitic acid (4). This was demonstrated later by Paltauf (5) who administered labeled natural (1-*O*-hexadecyl-*sn*-glycerol) and unnatural (3-*O*-hexadecyl-*sn*-glycerol) glycerol ether isomers to rats and showed that only the natural isomer was incorporated into intestinal mucosal lipids. Paltauf also showed that the exogenous alkylglycerol was further metabolized to plasmalogens in intestinal mucosal cells (5). Mangold (1) and Bandi *et al.* (6) reported similar findings by feeding rats glycerol ethers which contained unnatural (18:2, 18:3) alkyl chains. These works have found that dietary ether glycerolipids are not only incorporated into membrane ether lipids of intestinal mucosal cells, but a small portion is also transported to liver and incorporated into plasmalogens in that tissue (6). Reichwald and Mangold (7) fed rats 1-alkyl glycerols having unnatural, odd-numbered and polyunsaturated alkyl groups (13:0, 15:0, 17:0, 19:0, 19:1 and 19:2) and studied their incorporation into lipids of intestinal mucosal cells. They found that although all of these glycerol ethers are incorporated into alkylacyl-glycerophosphoethanolamine (GPE) and alkylacyl-glycerophosphocholine (GPC), only the glycerol ethers containing 17:0, 19:0 and 19:1 chain lengths are converted into the corresponding ethanolamine and choline plasmalogens. Weber (8) showed that dietary 1-*O*-dodecyl-*rac*-glycerol is incorporated into the alkylglycerolipids, but not into the plasmalogens, of all tissues in mice.

These studies from different laboratories established that mammals can utilize dietary glycerol ethers to make membrane ether lipids in different tissues. In recent years, the possible incorporation of exogenous ether lipids into tissue plasmalogens has assumed clinical significance because of the discovery of a number of genetic diseases involving ether lipid deficiency (9–11).

Heymans *et al.* (12) first reported the almost complete absence of ether lipids in the tissues of patients suffering from Zellweger cerebrohepatorenal syndrome. This deficiency of ether lipids is due to the absence of peroxisomes in the tissues of these patients (13). Peroxisomes have been shown to be the site for synthesis of ether lipids (14). It has been demonstrated that the activity of dihydroxyacetone phosphate acyltransferase (DHAPAT), which catalyzes the biosynthesis of the precursor of ether lipids, acyl dihydroxyacetone phosphate (acyl DHAP) (Fig. 1), is very low in the tissues of Zellweger patients (15–17). The activity of the enzyme catalyzing the synthesis of the ether bond, *i.e.*, alkyl DHAP synthase (Fig. 1), is also somewhat low in these patients (15,17,18). The deficiency of these peroxisomal enzymes, required for the *de novo* biosynthesis of the ether bond in ether glycerolipids, results in a decreased amount of plasmalogens in the tissues of Zellweger patients. The same enzymes have also been shown to be deficient in a number of other genetic diseases involving peroxisomal disorders, such as neonatal adrenoleukodystrophy (19), infantile Refsum disease (20), rhizomelic chrondrodysplasia punctata (9) and others (21). On the other hand, the activity of the key enzyme necessary for the utilization of exogenous 1-*O*-alkyl-*sn*-glycerol, *i.e.*, alkyl glycerol kinase (Fig. 1), was shown not to be deficient in these patients (15). Considering that the enzymes that catalyze the conversion of 1-alkyl-*sn*-glycerol-3-phosphate to membrane ether glycerolipids and plasmalogens are localized not in peroxisomes (14,22) but in the endoplasmic reticulum (Fig. 1), it is expected that exogenous alkyl glycerols might be incorporated into tissue ether lipids in these patients. This has been shown to be the case in cultured skin fibroblasts

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Abbreviations: DHAP, dihydroxyacetone phosphate; DHAPAT, dihydroxyacetone phosphate acyltransferase; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine.

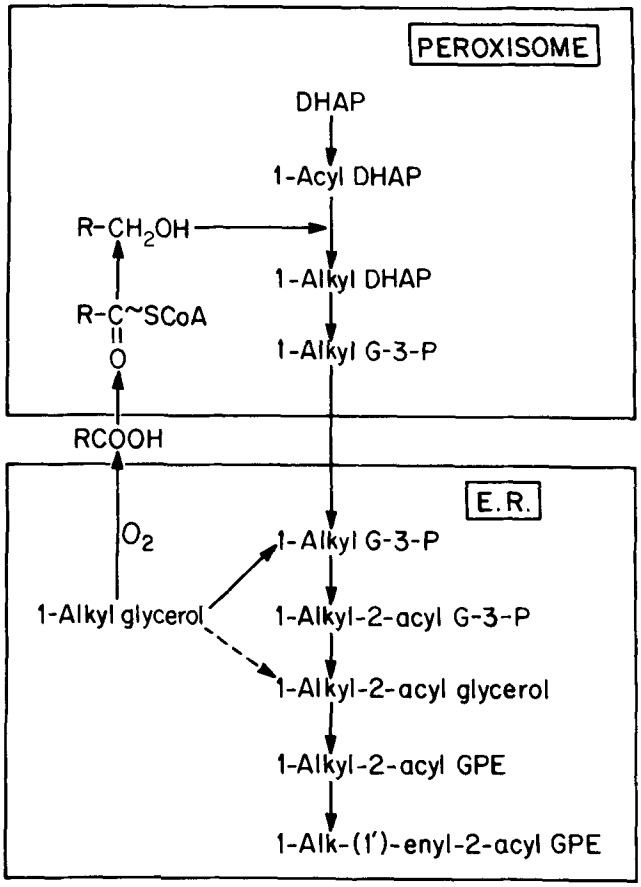

FIG. 1. Subcellular compartmentation of the enzymatic reactions leading to the biosynthesis of alkyl glycerol ethers and plasmalogens.

obtained from Zellweger patients where the labeled alkyl glycerols, added to the culture medium, are incorporated into cellular plasmalogens, but the labeled long-chain alcohols are not (23,24).

ORAL ETHER LIPID THERAPY IN PATIENTS WITH ETHER LIPID DEFICIENCY

The findings summarized above led us to hypothesize that oral administration of ether lipids to patients with peroxisomal disorders might rectify the tissue ether lipid deficiency (15,25). Our group has given several patients with peroxisomal disorders an ether lipid suspension containing 400 mg 1-*O*-octadecyl-*sn*-glycerol (batyl alcohol) and 200 mg sodium deoxycholate in 100 mL of water. Each patient received 5–10 mg/kg daily of batyl alcohol (25). Case histories for three patients are described below.

Patient 1. NR was born on May 26, 1983, following a full-term gestation. Birth weight was 3.13 kg (50%), length 54 cm (95%) and head circumference 34.5 cm (50%). He was evaluated at 7 weeks of age because of failure to thrive and was noted to have hypotonia and enlarged anterior and posterior fontanels with diastasis of the sagittal suture. Hepatomegaly was present at age 4 months and serum aminotransferase levels were elevated. Percutaneous liver biopsy showed abnormal lobular architecture with fibrous septa extending from portal tracts into the surrounding parenchyma. By 8 months of age he could not visually fix on objects and had wandering nystagmus. Ophthalmologic evaluation revealed bilateral punctate cortical and plaque-like nuclear changes, optic atrophy and retinal degeneration.

A skin biopsy was performed at 1 year of age and fibroblasts were cultured, harvested and assayed for the activity of the peroxisomal membrane enzyme, dihydroxyacetone phosphate acyltransferase (DHAPAT). The activity was 30% of control. Plasma levels of C_{26} very long-chain fatty acids and the ratio of C_{26}/C_{22} assayed at 20 months of age were elevated in a pattern diagnostic of neonatal adrenoleukodystrophy.

Dietary supplementation with batyl alcohol was started in August, 1984, and continued through May, 1987. The child is currently enrolled in special education classes with a level of development equivalent to a young toddler. He has not yet developed clinical or laboratory evidence of adrenal insufficiency (normal cortrosyn stimulation test at age 8 1/2 years).

Patient 2. EZ was born at 40 weeks gestation by emergency cesarean section on September 28, 1982, because of fetal distress and maternal hypertension. Birth weight was 2.5 kg and length 53 cm. At 6 months of age she was evaluated for failure to thrive, hypotonia and hepatomegaly. A liver biopsy was performed and sections of the liver showed bridging fibrosis and paucity of intrahepatic bile ducts without cholestasis.

She was re-evaluated at age 2 years because of failure to thrive, delayed development, seizures, hypotonia and persistent hepatomegaly with elevated levels of serum aminotransferases. Her facies was not dysmorphic, although there was a high-arched palate. Because of these findings, fibroblasts cultured from a skin biopsy were assayed for DHAPAT. The activity of fibroblast DHAPAT was 0.125 nmol/min/mg protein (control value = 0.2 nmol/min/mg protein). The reference non-peroxisomal enzyme glycerol-3-phosphate acyltransferase had values of 0.89 nmol/min/mg (control = 0.81 nmol/min/mg protein). Erythrocyte plasmalogen content was assayed at age 3 years and was abnormal (Table 1).

She was given a suspension of batyl alcohol from July, 1985, until February, 1989. There were no adverse effects.

TABLE 1

Erythrocyte Ethanolamine Plasmalogen Concentration Before and After Administration of Batyl Alcohol[a]

	Ethanolamine plasmalogen (% of total phospholipids)		
	Patient no. 1	Patient no. 2	Patient no. 3
Pretreatment value	4.0	1.3	4.1
Post-treatment value	10.6	11.2	7.5

[a]Erythrocyte phospholipid composition was analyzed by two-dimensional thin-layer chromatography as previously described (30). The percentage of ethanolamine plasmalogen is shown here. Most of the other phospholipid percentages were the same as normal except for phosphatidylethanolamine, which was increased. The ethanolamine plasmalogen contents of normal subjects varies from 10% to 15% of the total phospholipids. For further details, see text and ref. 25.

She is presently attending a school for developmentally handicapped children and is beginning to walk. There is marked speech delay.

Patient 3. CB was born on January 14, 1986, following full-term gestation. Hypotonia and strabisimus were present at birth. He was evaluated at age 4 months because of failure to thrive, and was noted to have a dysmorphic facies and hepatomegaly. Plasma very long chain fatty acid levels were elevated and the activity of fibroblast DHAPAT was determined to be 40% of control value. These findings supported the diagnosis of neonatal adrenoleukodystrophy.

A cortrysn stimulation test was performed in March, 1988. The result was abnormal and hydrocortisone replacement therapy was started. In October, 1988, he developed a diffuse, erythematous skin rash, associated with elevation of plasma phytanic acid.

An oral suspension of batyl alcohol was prescribed in October, 1986, and continued to January, 1989. The erythrocyte plasmalogen content was measured before and after treatment with batyl alcohol. The results are summarized in Table 1.

At the present time, he is attending a special education program for hearing-impaired and vision-impaired children. He has marked developmental delay and is not yet able to walk.

The results presented in Table 1 show that erythrocyte plasmalogen levels are increased in these patients after oral feeding of batyl alcohol for a few months. In one patient, who was under oral batyl alcohol therapy for three months, the chain length distribution of the alk-1′-enyl moiety of ethanolamine plasmalogens from erythrocytes was analyzed. An increase in the 18:0 moiety (68% *vs.* 50% in normal controls) was observed, suggesting that the dietary batyl alcohol was absorbed, metabolized and incorporated into the erythrocyte plasmalogens of this patient.

Administration of oral ether lipids represents a potential treatment for patients with peroxisomal disorders. Although none of the patients reported in this series have the severe Zellweger phenotype associated with multiple peroxisomal enzyme deficiency, they all share clinical features typically seen in patients with peroxisomal dysfunction: failure to thrive, delayed development, hypotonia, facial dysmorphia and hepatomegaly. Each of these patients has a documented defect of plasmalogen synthesis and oxidation of fatty acids. These infants have exhibited subjective improvement of nutritional status, liver function, retinal pigmentation and motor tone and repletion of deficient red cell ethanolamine plasmalogens since receiving batyl alcohol. However, it is not possible to separate these changes from the natural history of untreated disease. Wanders *et al.* (26) have documented increasing plasmalogen levels in Zellweger's syndrome as a function of age. This may be due to the intake of natural alkylglycerols present in food. Horrocks (27) estimated that the average adult consumes 10–100 mg of batyl alcohol each day.

INCORPORATION OF DIETARY ALKYLGLYCEROL INTO PLASMALOGENS OF DIFFERENT TISSUES IN RATS

The increase in the plasmalogen level in erythrocytes of patients after feeding batyl alcohol indicated that dietary ether lipids may also be incorporated into different tissues of these patients. However, a direct demonstration of such an increase in tissues of these patients was not feasible. There are also no reports of incorporation of dietary ether lipids into plasmalogens of different tissues of animals except in intestinal mucosa (1,5) and, to a small extent, in liver of rats (6). By contrast, there are reports of nonincorporation of exogenous tracer doses of labeled ether lipids into tissue plasmalogens of animals (28,29). Therefore, to determine whether dietary ether lipids are utilized to synthesize membrane ether lipids of different tissues, we administered 1-*O*-heptadecyl-*sn*-glycerol to young rats. The odd chain heptadecyl moiety is practically absent in normal ether glycerolipids, but it is very similar to the alkyl and alk-1′-enyl groups of tissue lipids that are mainly composed of 16:0, 18:0 and 18:1 chains. By feeding rats (19-d-old) 1-*O*-heptadecyl-*rac*-glycerol (1–2% mixed with food) for 5 to 10 days, we found a very high (46–60%) incorporation of the C_{17}-moiety at the C-1 position of ethanolamine plasmalogens in most tissues of these rats (30). However, the total plasmalogen content of the tissues did not increase. These results show that dietary alkyl glycerol ethers are not only absorbed and converted to plasmalogens in intestinal tissue, but are also transported to other organs where they are metabolized and deposited as membrane plasmalogens. The incorporation of the exogenous ether lipids into tissue plasmalogens is quite high, much higher than reported previously (7). This is probably due to our use of young growing rats for these experiments, as opposed to the use of adult rats by other authors. Recently, Blank *et al.* (31) reported a similar observation. These authors fed 1-*O*-alkyl-2,3-diacetyl-*sn*-glycerol, containing 65% 18:1 and 17% 16:1 alkyl groups, to rats, and found an increase in the 18:1 group of alkyl and alk-1′-enyl side chains of ether phosphoglycerides in liver, kidney and lung. There was an increase in the alkyl glycerol ether content but, as we have found, no change in the plasmalogens content of these tissues (31). We further investigated the mode of incorporation of dietary ether lipids into tissue lipids by feeding different precursors of ether lipids, such as heptadecanoic acid, heptadecanol, 1-*O*-heptadecyl-*sn*-glycerol and 3-*O*-heptadecyl-*sn*-glycerol, to young rats. The results are summarized in Figure 2. It is seen that 1-*O*-heptadecyl-*sn*-glycerol is the best precursor, whereas its unnatural optical isomer, 3-*O*-heptadecyl-*sn*-glycerol, is incorporated only to a very small extent. Apparently, the *sn*-3 isomer is oxidized to heptadecanoic acid (the mono-oxygenase oxidizing the ether bond is non-stereospecific, see ref. 32) which is further utilized. This conclusion is supported by the higher percentage of incorporation of heptadecanol and heptadecanoic acid than of the *sn*-3 isomer (Fig. 2). The relative incorporation rates, *i.e.* 1-*O*-heptadecyl-*sn*-glycerol $\gg$ heptadecanol > heptadecanoic acid > 3-*O*-heptadecyl-*sn*-glycerol (Fig. 2), suggest that the pathway shown (Fig. 1) for the incorporation of different precursors into ether lipids is operative in all tissues. These results and results from other laboratories (7,8,31) indicate that most exogenous long chain ether lipids incorporate into alkylglycerol ether lipids, but the subsequent conversion to plasmalogens occurs only in lipids containing alkyl chain lengths between C_{15} and C_{19} (saturated or monounsaturated). Thus, it appears that, physiologically, the 1′-alkenyl chain lengths are restricted to a narrow range,

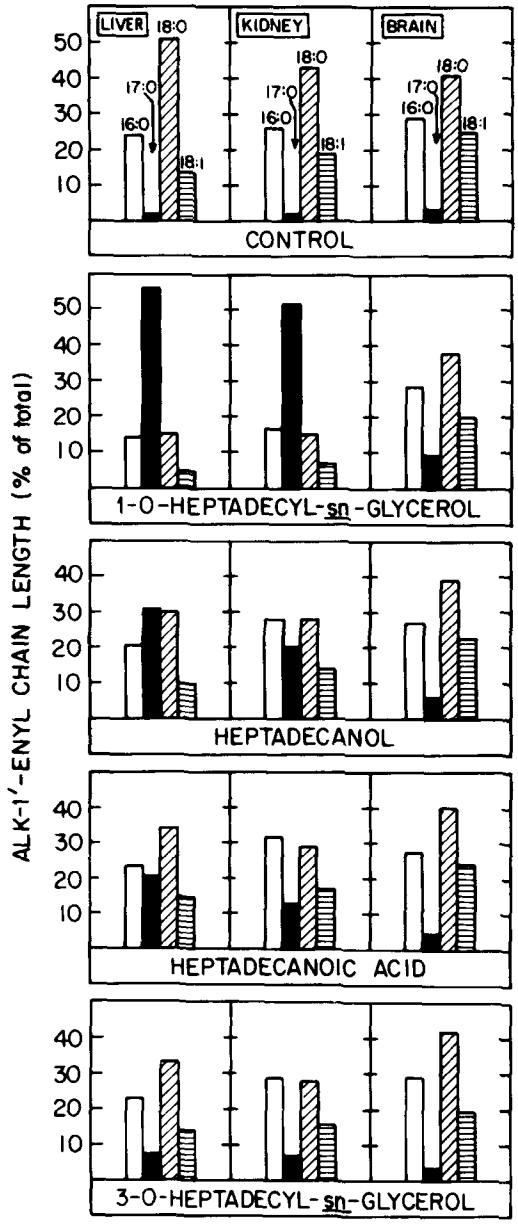

FIG. 2. Incorporation of dietary 1-*O*-heptadecyl-*sn*-glycerol, heptadecanol, heptadecanoic acid and 3-*O*-heptadecyl-*sn*-glycerol into ethanolamine plasmalogen of the tissues of 19-day-old rats. Rats were fed either powdered food mixed with the indicated compounds (1%, w/w) or only the powdered food alone (control group) for 5 days, and then the chain length distributions of the alk-1'-enyl group at C-1 position of ethanolamine plasmalogen of liver, kidney and brain were analyzed, as described previously (30).

not only by the specificity of the acyl-CoA reductase (33), but also by the chain length specificity of the dehydrogenase which converts 1-*O*-alkyl phosphoglycerides to 1-*O*-[1'-alkenyl]phosphoglycerides (34). The nature of the ether lipids transported from intestine to liver or to other organs is a matter of speculation. However, it is plausible that esterified alkylglycerols (ether analogs of triglycerides) are transported from the intestine (2–4) and liver to other organs and that these alkylglycerol derivatives are utilized after partial or complete hydrolysis of the ester bonds. Neutral lipids, whose chromatographic migration rate is similar to alkyldiacylglycerol, were found to be present in the liver and kidney of animals who were chronically fed heptadecylglycerol (Das, A.K., and Hajra, A.K., unpublished observation; see also ref. 31).

The results of our animal feeding experiment show that dietary ether glycerolipids are effectively utilized to synthesize membrane ether lipids in most tissues. However, in brain the incorporation of the exogenous ether lipid precursor was always very low (ref. 30 and Fig. 2). This could possibly be due to either a low rate of ether lipid synthesis in brain after myelination is complete, or to the fact that the ether lipid precursors cannot cross the blood-brain barrier. To resolve this question, ether lipids should be fed to rats before the start of myelination (<10 d old). It is difficult, however, to feed large amounts of ether lipids to these suckling rats. Therefore, we fed pregnant female rats powdered food containing 0.5% (w/w) 1-*O*-heptadecyl-*sn*-glycerol on the fifth day before delivery, and continued until the fourteenth day after the pups were born. The babies were allowed to suck the mother's milk which, when analyzed, was found to contain 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols. When the tissues of the babies (14-d-old) were analyzed, 28% of liver and 17% of kidney alk-1'-enyl groups of ethanolamine plasmalogen were found to contain the 17:0 moiety. These values were lower than those obtained by direct feeding of heptadecylglycerol (30). In brain, a low (2%) incorporation of 17:0 in the alkenyl chain was also observed. It is not clear whether this low incorporation into brain lipid is due to the blood-brain barrier or to the lower consumption of heptadecylglycerol by the babies. In another experiment, we found no incorporation of the 17:0 moiety in the tissues of newborn babies whose mothers had been fed 1-*O*-heptadecylglycerol for 20 days during pregnancy, though the mothers' tissues contained a high percentage (40–60%) of the heptadec-1'-enyl moiety in the ethanolamine plasmalogens. This indicates that ether lipids are not transported across the placenta from mother to fetus.

From these results, it is evident that dietary ether lipids can be directly utilized by mammals to synthesize membrane alkyl glycerolipids and plasmalogens in most tissues. Although the utilization of the exogenous ether lipids for the formation of brain plasmalogens is somewhat questionable, there was a low but reproducible incorporation of 1-*O*-heptadecyl-*sn*-glycerol into brain ether lipids (ref. 30, Fig. 2). It is possible that the exogenous ether lipids would be more effectively utilized if there were no endogenous formation of ether lipids. Increasing the dose might result in higher incorporation of ether lipids in all tissues of the patients. Oral ether lipid therapy may never relieve all of the syndrome associated with these diseases because of multiple peroxisomal enzyme defects (10,22). But, in mild cases, where the main problem is the deficiency of ether lipids, oral ether lipid therapy may prove helpful. Alkyl glycerols are fairly innocuous compounds which have been shown to be nontoxic even at high doses (1), and no harmful effects are expected when these compounds are chronically administered to the patients. Oral ether lipid therapy should be attempted to alleviate some of the symptoms associated with these peroxisomal disorders.

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ARTICLE

Characterization of a Cytosolic Protein in Rat Liver Inhibiting Neutral Cholesteryl Ester Hydrolase

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Neutral cholesteryl ester hydrolase activity (EC 3.1.1.13) present in microsomes isolated from lactating rat mammary glands was found to be inhibited by a factor (or factors) occurring in the cytosolic fraction of male rat liver. The inhibitor was heat-labile, non-dialyzable, destroyed by proteolysis, and was stable following preparation of an acetone/diethyl ether powder of the cytosolic fraction. The protein also inhibited the activity of hormone-sensitive lipase (HSL) (from bovine adipose tissue) and esterase from *Candida cylindracea*, but seemed to be more active against the neutral hydrolase found in rat liver microsomes. For the mammary gland microsomal cholesteryl ester hydrolase, the extent of the inhibitory effect was dependent on the concentration of the cytosolic protein, 50% inhibition being achieved by about 100 μg of cytosolic protein, and on the method of initiating the enzyme assay. Kinetic analysis indicated that, under circumstances where the reaction was initiated by the addition of substrate, the inhibition was characterized as "uncompetitive." When an inhibitor/substrate complex was allowed to form in the absence of enzyme, an element of "competitive" inhibition was introduced into the reaction. Food withdrawal reduced the activity of the inhibitor in liver by 56%, but activity was fully restored by short-term re-feeding. In contrast, feeding a diet high in fat led to a 34% increase in activity. The present findings suggest that the inhibitory factor(s) may be involved in the regulation of the hydrolysis of cholesteryl esters in the liver and also in other cell types. *Lipids* 27, 406–412 (1992).

Hepatic cholesteryl esters, which derive primarily from the uptake and degradation of plasma lipoproteins, are hydrolyzed in liver cells through the action of the enzyme cholesteryl ester hydrolase (CEH). The resulting free cholesterol, as well as the endogenously synthesized sterol can be catabolized, re-esterified for storage, or secreted from the liver. In addition, recent studies on CEH regulation suggest that cholesteryl ester metabolism may play a role in steroid hormone synthesis (1–3). Thus, free and esterified cholesterol are subject to constant turnover, and the enzyme CEH plays an important role in controlling the supply of free sterol from its storage form. At least three CEH activities have been described that are located in different hepatic subcellular compartments (4–7). It is known that lysosomal CEH, which has an optimum pH in the acidic range, is required for the hydrolysis of exogenous cholesteryl ester, internalized as lipoproteins into cells by receptor-mediated endocytosis (8–10). Recent research has indicated that the neutral CEH of macrophages (11), adrenal cortex (12), corpus luteum (13), mammary gland (14) and heart (15) are identical to the hormone-sensitive lipase of rat adipose tissue, and it has been suggested that all of the neutral CEH in tissues are the same enzyme (16). There is now considerable evidence that these enzymes are under hormonal control and that this hormonal activation is mediated by a covalent phosphorylation of the enzyme catalyzed by cyclic adenosine monophosphate (AMP)-dependent protein kinase (16,17). The activity of the cytosolic neutral CEH in the liver is also reported to be increased by cyclic AMP-dependent phosphorylation (18), but is extremely low in contrast to the enzyme of adipose tissue and adrenal cortex (12). This is in spite of the fact that the liver is thought to be the major site of cholesteryl ester hydrolysis in the rat.

The liver does, however, contain a neutral CEH that is activated by bile salts at levels higher than their critical micelle concentration (19), and it is possible that the effect of these bile salts could be to increase the efficiency with which cholesteryl ester substrate is presented to the enzyme (20,21). There is considerable evidence that the activity of various CEH is markedly affected by the manner in which the substrate is presented to the enzyme. Cholesteryl esters are insoluble in aqueous media and the substrate is usually presented either as a solution in ethanol or acetone, or suspended in a mixed micelle with bile salts and phospholipids (20). This raises the possibility that within the cell the activity of the neutral CEH may also be controlled by the manner in which the enzyme interacts with its substrate. An inhibitor of lysosomal acid CEH, which does not affect the neutral hydrolase, has been reported to exist in the cytosolic fraction of rat liver (22) and in cultured fibroblasts (23), and more recently this same enzyme has been shown to be inhibited by apolipoprotein A-I (24).

We have re-investigated the cytosolic fraction of rat liver and have now shown that this fraction does contain a highly active inhibitor of neutral CEH that appears to function by interfering with the presentation of substrate to enzyme.

MATERIALS AND METHODS

Materials. [1-^{14}C]Oleic acid (1.95 GBq/mmol) and [9,10(n)-^{3}H]oleic acid (74-370 GBq/mmol) were purchased from Amersham International (Amersham, Bucks, U.K.). Cholesteryl [9,10(n)-^{3}H]oleate was synthesized from cholesterol and [9,10(n)-^{3}H]oleic acid by a modification of the method of Swell and Treadwell (25). [9,10(n)-^{3}H]Oleic acid (10 mg) was dissolved in oxalyl chloride (0.5 mL) and heated at 50°C for 24 h. The excess reagent was removed in a stream of nitrogen and a solution of cholesterol (30 mg) and pyridine (100 μL) in dry toluene (2 mL) was added. The mixture was kept at 50°C overnight and the excess solvent and reagents again evaporated off. The final product was purified by thin-layer chromatography (TLC) and gave a product with specific activity of (42 mCi/mmol). All biochemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) or from BCL (Lewes, Sussex, U.K.).

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; AMP, adenosine monophosphate; BSA, bovine serum albumin; CEH, cholesteryl ester hydrolase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*(2-aminoethyl)tetraacetic acid; HSL, hormone-sensitive lipase; MES, 2-(*N*-morpholino)ethanesulfonic acid; M_r, relative molecular weight; NEM, *N*-ethylmaleimide; TRIS, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography.

Preparation of the inhibitory factor from rat liver. Male Wistar rats (280–330 g) were maintained in an alternating light cycle of 12 h (light from 0800) and were fed *ad libitum* on a CRM(X) breeding diet (Special Diet Services, Manea, Cambridgeshire, U.K.). Starved rats were deprived of food for 24 h or 48 h, and starved/re-fed rats were allowed food for 2 h after 48 h starvation. One group of rats was given a high-fat diet (26), comprising potato crisps, cheese crackers and chocolate chip cookies, in addition to chow, for 2 wk prior to use. Animals were killed by cervical dislocation at 0930 (except where indicated in the text) and the livers excised and homogenized (Polytron, Kinematica, Luzern, Switzerland; PMA20N probe, 17000 rpm, 30 sec) in 4 volumes of ice-cold medium consisting of 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), 50 mM tris(hydroxymethyl)aminomethane (TRIS)/HCl buffer pH 7.2, 1 mM ethylenediaminetetracetic acid (EDTA), 1 mM ethyleneglycol-*bis*(2-aminoethyl)tetraacetic acid (EGTA), and 1 mM dithiothreitol (Buffer A). The homogenate was centrifuged at 10,000 × *g* for 10 min and the resulting supernatant centrifuged at 100,000 × *g* for 60 min. The clear supernatant below the floating fat layer was carefully removed and either stored in liquid nitrogen or converted into an acetone/diethyl ether powder by addition to 10 volumes of ice-cold acetone. The resultant precipitate was recovered by centrifugation (5,000 × *g*, 5 min) and washed with cold acetone and diethyl ether before being dried in a vacuum desiccator. The dried powder was stored at −20°C. Before use it was extracted in a volume of Buffer A equivalent to that of the original solution and dialyzed or passed through a column of Sephadex G15 (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Partial purification of the inhibitory material. The 100,000 × *g* supernatant was acidified to pH 5.2 with 1 M acetic acid and after standing for 30 min in ice was centrifuged 100,000 × *g* for 15 min. The pH of the resultant supernatant was re-adjusted to pH 7.2 with 1 M TRIS, and ammonium sulfate was added to bring the solution to 70% saturation. After 60 min in ice, the suspension was again centrifuged (10,000 × *g*, 15 min) and the precipitate dissolved in a minimum volume of Buffer A. An acetone/diethyl ether powder was prepared from this solution as detailed above.

Gel filtration chromatography. The 100,000 × *g* supernatant from the homogenized liver or the dialyzed extract of the acetone/diethyl ether powder (3 mL) was applied to a column (24 × 900 mm) of Sephacryl S-300HR (Pharmacia) equilibrated in the Buffer A. Fractions of 2.5 mL were collected and 140 μL of every second fraction assayed under standard conditions for activity with respect to inhibition of the neutral CEH in a mammary microsomal preparation.

Interaction with phenyl sepharose. A sample of phenyl sepharose (Pharmacia) was equilibrated with Buffer A in a small column. The column was centrifuged to remove excess buffer and the phenyl sepharose (100 mg) added to small tubes together with the solution containing inhibitory activity (120 μg). After 60 min in ice, the tubes were centrifuged (9,000 × *g*, 5 min) and the supernatant assayed for remaining inhibitory activity.

Heat stability. To test the heat stability of the inhibitory material, an extract of the acetone/diethyl ether powder was heated at 95°C for 5 min. After centrifugation (9,000 × *g*, 5 min) to remove precipitated protein, the remaining inhibitory activity was assayed.

Influence of proteolysis. An extract of the acetone/diethyl ether powder (400 μg) was incubated at 37°C with trypsin (30 μg). After 15 min, proteolysis was terminated by the addition of soybean trypsin inhibitor (60 μg) and the resultant solution assayed for inhibitory activity. Control incubations where the trypsin inhibitor was added before the trypsin were also included.

Influence of N-ethyl maleimide. A sample of the acetone diethyl ether powder extract was passed through a column of Sephadex G-15, equilibrated in 50 mM TRIS/50 mM MES buffer (pH 7.0), to remove dithiothreitol and the resultant solution incubated at 37°C with *N*-ethyl maleimide (2 mM). After 15 min, the reaction was stopped by the addition of dithiothreitol (5 mM) and the extract again passed through a small column of Sephadex G-15 equilibrated with Buffer A. The efficiency of the inhibitor in the acetone/diethyl ether powder was assayed before and after this treatment.

Preparation of rat mammary and liver microsomes. Microsomes were prepared from the mammary glands of rats in mid-lactation as previously described (27) and from the liver of virgin female rats according to the method of Polokoff *et al.* (28). They were stored at −80°C as a suspension in Buffer A.

Assay of CEH. Neutral CEH activity in the mammary microsomal membranes (100 μg) was routinely assayed using Buffer A containing bovine serum albumin (BSA) (2%, w/v). The inhibitory fraction (10–120 μg) was included in the assay mixture (final volume 200 μL) and the reaction was initiated by the injection of an ethanolic solution of cholesteryl [9,10(n)-^{3}H]oleate (20 nmol, 200,000 dpm). Where indicated, the reaction was also initiated by the addition of the microsomal membranes, suspended either in Buffer A or in a solution of BSA in Buffer A, to the incubation tubes containing all of the other assay constituents. In all instances the final assay mixtures were identical. After 20 min at 37°C the reaction was terminated and the extent of hydrolysis of the substrate determined as previously described (27). Assays were performed in triplicate and appropriate controls without microsomes were incubated concurrently to correct for background and possible CEH activity in the cytosolic fractions. Where indicated, taurocholate was present at a final concentration of 1 mM. Where the activity of the CEH in the liver microsomes was assayed, the microsomal protein concentration was kept below 250 μg/mL and the incubation period was increased to 30 min (7).

Protein measurement. The protein concentrations were determined by the dye-binding technique of Bradford (29) using crystalline BSA as standard.

RESULTS

The neutral CEH activity in both mammary and liver microsomes was markedly inhibited when the cytosolic fraction from liver was included in the assay (Figs. 1 and 2). Although the inhibitory effect was dependent on the concentration of cytosolic protein, total inhibition of the enzyme activity was not observed (Fig. 1). The reduction in esterase activity was not due to dephosphorylation of the enzyme by phosphatases in the liver cytosolic fraction (16) since incubation of the mammary microsomes

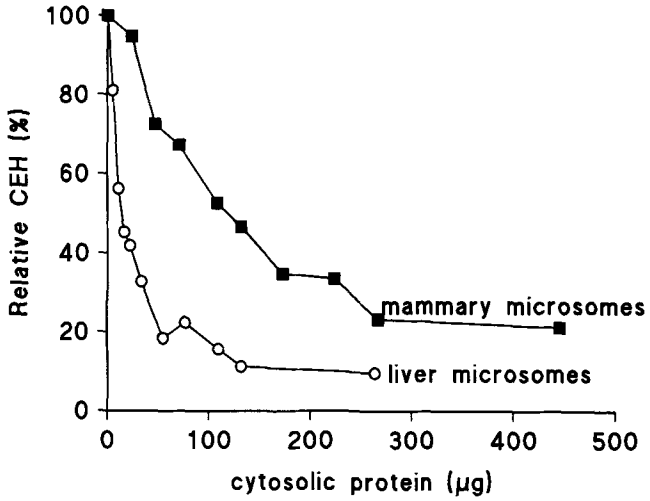

FIG. 1. Inhibitory effect of the cytosolic fraction from rat liver on the CEH in microsomes isolated either from lactating rat mammary glands or from rat liver. The assay for CEH activity was performed, as described in Materials and Methods, in the presence of varying amounts of an extract of an acetone/diethyl ether powder of rat liver cytosol. The mammary microsomal enzyme hydrolyzed 486 pmol of cholesteryl oleate per min per mg of protein, while the liver microsomal enzyme hydrolyzed 90.6 pmol cholesteryl ester per min per mg of protein.

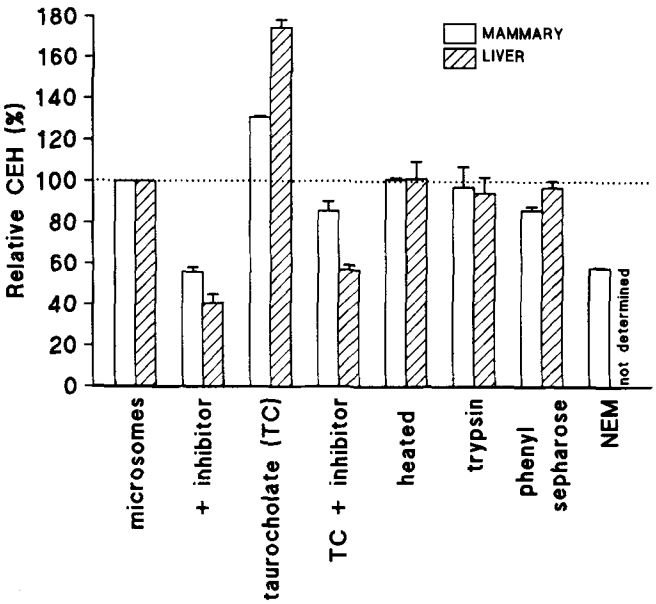

FIG. 2. The effect of various treatments on the inhibition of CEH by hepatic cytosolic factors. The assay for CEH activity in both mammary (100 μg) and liver (23.5 μg) microsomes was performed, as described in the Materials and Methods section, in the presence and absence of a dialyzed extract of an acetone/diethyl ether powder of liver cytosol (enzyme/inhibitor = 1:1 for mammary, 1:1.9 for liver). Where indicated, the inhibitor was heated, or was treated with *N*-ethyl-maleimide (NEM), phenyl sepharose, or trypsin as detailed in Materials and Methods. The assay was also performed in the presence of taurocholate (1 mM) with and without the inhibitor. The results are the means ± SEM of triplicate determinations and are expressed relative to the activity (100%) of the microsomal CEH. The mammary enzyme hydrolyzed 548.7 ± 8.93 pmol of cholesteryl oleate per min per mg protein while the activity of the liver enzyme was 73.3 ± 3.1 pmol cholesteryl oleate hydrolyzed per min per mg of protein.

TABLE 1

Partial Purification of Cytosolic Inhibitor[a]

	Total protein (mg)	Specific activity (μg protein for 50% inhibition)	Purification
Homogenate	14300	770.5	1.0
pH 5.2 supernatant	5555	271.0	2.8
Acetone precipitate	3141	66.7	11.6

[a]Rat livers (100 g) were homogenized in 4 vol of ice-cold medium consisting of 50 mM TRIS, 50 mM MES, pH 7.2, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol. The pH 5.2 supernatant and the acetone precipitate were then prepared as detailed in Materials and Methods. The specific activity was taken as the amount of protein necessary for 50% inhibition of the neutral CEH activity in 100 μg of mammary gland microsomal membranes.

with a sample of partially-purified rat liver phosphoprotein phosphatase (30) had no effect on its CEH activity. The inhibitory activity was retained on dialysis but was heat-labile, being totally destroyed by treatment of the cytosolic fraction at 95°C (Fig. 2). It was also completely abolished following incubation of the material with trypsin (Fig. 2). Adjustment of the pH of the cytosol to 5.2 did not cause precipitation of the inhibitory activity (Table 1). It also survived the preparation of an acetone/diethyl ether powder (Table 1). This treatment, which removes endogenous lipid, indicates that inhibition was not an artefact arising from alterations in the specific activity of the substrate by cholesteryl esters in the fraction. The bulk of the cytosolic activity was bound to phenyl sepharose indicating its hydrophobic characteristics (Fig. 2). The neutral CEH was stimulated by low concentrations (1 mM) of taurocholate, but the presence of the bile salt did not prevent the inhibition produced by the cytosolic material (Fig. 2). The cytosolic factors also inhibited the activity of bovine adipose tissue hormone-sensitive lipase (HSL) (reduced from 270.17 nmol/min/mg protein to 73.4 nmol/min/mg protein by 125 μg of cytosolic protein) and the activity of the CEH from *Candida cylindracea* (EC 3.1.1.13) (reduced from 2194 nmol/min/mg protein to 1075 nmol/min/mg protein by 62.6 μg of cytosolic protein).

Variation in the method of initiating the hydrolysis of the cholesteryl esters affected both the rate of the reaction and the extent to which it was inhibited by the cytosolic factors. When the reaction was initiated by the addition of the mammary gland microsomes, either as a suspension in buffer or as a suspension in a solution of BSA, the rate of hydrolysis by the neutral CEH was slower than that observed under the normal conditions of the assay (*i.e.*, initiation by injection of an ethanolic solution of the substrate). Nevertheless, when the mixture of microsomes and BSA was used to initiate the reaction, the inhibitory effect was significantly enhanced (Table 2). Similar, but less marked effects were obtained with the liver microsomal enzyme.

Injection of an ethanolic solution of cholesteryl oleate into aqueous medium results in the formation of a finely dispersed suspension of the substrate. Enhancement of the precipitation of this suspension would not, however, explain the increased efficiency of inhibition because the presence of the inhibitor actually diminished such sedimentation (Table 3). Initiation of the reaction in this manner allowed the inhibitor to react with the substrate, in

TABLE 2

Neutral CEH Activity. Effect of Method of Initiating Assay[a]

Initiation	Neutral cholesteryl esterase activity (pmol/min/mg protein)		Percentage activity remaining
	Control	Plus inhibitor	
(a) Substrate	548.72 ± 8.93	242.09 ± 12.49	44.2 ± 2.28
(b) Microsomes	339.80 ± 11.16	128.85 ± 6.78	37.9 ± 1.99
(c) Microsomes plus BSA	380.40 ± 11.37	50.30 ± 1.25	14.9 ± 1.0

[a]The reaction was initiated by addition of (a) an ethanolic solution of cholesteryl [9,10(n)-^{3}H]oleate (20 nmol, 200,000 dpm), (b) a suspension of mammary microsomes (100 μg), or (c) a suspension of mammary microsomes (100 μg) in bovine serum albumin (4 mg) to the remaining constituents of the assay. Where indicated, the assay contained 99.6 μg of an extract of an acetone/diethyl ether powder of rat liver cytosol. The mixtures were incubated for 20 min before the reaction was terminated and the extent of hydrolysis determined as described in Materials and Methods. The results given are the means ± SEM for triplicate determinations and are expressed in terms of the pmol of cholesteryl oleate hydrolyzed per min per mg microsomal protein.

TABLE 3

Influence of Hepatic Cytosolic Protein on Amount of Cholesterol Oleate Retained in Aqueous Solution[a]

Cytosolic protein (μg)	Cholesteryl ester in supernatant (nmol)
None	9.02 ± 0.12 (45.1%)
22.0	14.70 ± 0.24 (73.5%)
43.9	14.52 ± 0.35 (72.6%)
65.9	14.64 ± 0.02 (73.2%)

[a]An ethanolic solution of cholesteryl [9,10(n)-^{3}H]oleate (10 μL, 20 nmol, ≈200,000 dpm) was added to an aqueous solution containing 50 mM TRIS, 50 mM MES, pH 7.2, 2 mM dithiothreitol, and varying amounts of the extract of an acetone/diethyl ether powder of rat liver cytosol in a total volume of 200 μL. After 15 min at 37°C, the solution were centrifuged (9,000 × *g*, 2 min) and the radioactivity in duplicate aliquots of the supernatant determined. The results are the means ± SEM of triplicate determinations.

the absence of microsomes and BSA, prior to the assay, and this may be the explanation for its increased efficiency. The results from Table 3 would suggest that around 5.6 nmol of cholesteryl ester could be bound by the inhibitory fraction at 37°C, effectively reducing the amount of cholesteryl ester available to the microsomal CEH.

Kinetic analysis. A Lineweaver-Burk plot of the data obtained with the mammary microsomal preparation demonstrated that, when the reaction was initiated by the addition of substrate, the presence of the inhibitory activity produced a plot of 1/V against 1/S that was parallel to the plot obtained in its absence (Fig. 3a). Thus, both the K_m and the V_{max} were reduced (Table 4) indicating that inhibition was "uncompetitive" (31). However, when the reaction was initiated by addition of microsomes, the slope of the line, derived in the presence of the inhibitor, was much steeper than that observed in its absence (Fig. 3b). The increase in K_m and the decrease in V_{max} (Table 4) was consistent with a "mixed" type of inhibition (31). Essentially the same phenomena were observed with the liver microsomal enzyme when the method of initiation of the reaction was varied (Table 4).

TABLE 4

CEH Activity in Microsomes Isolated from Rat Mammary Gland and Liver. Variation of Kinetic Parameters with the Method of Initiation of the Reaction in the Presence and Absence of the Liver Cytosolic Inhibitor[a]

Enzyme source	Initiation by addition of	K_m (μM)		V_{max} (pmol/min/mg)	
		Control	Inhibited	Control	Inhibited
Mammary gland	Microsomes	9.71	33.78	198.2	34.3
	Substrate	20.68	10.18	1060.0	524.3
Liver	Microsomes	30.68	35.36	78.0	50.3
	Substrate	26.27	17.96	70.0	48.1

[a]For the mammary gland enzyme the kinetic parameters were calculated from the data shown in Figures 3a and 3b. For the liver microsomal enzyme the parameters were calculated from similar plots, which are not shown.

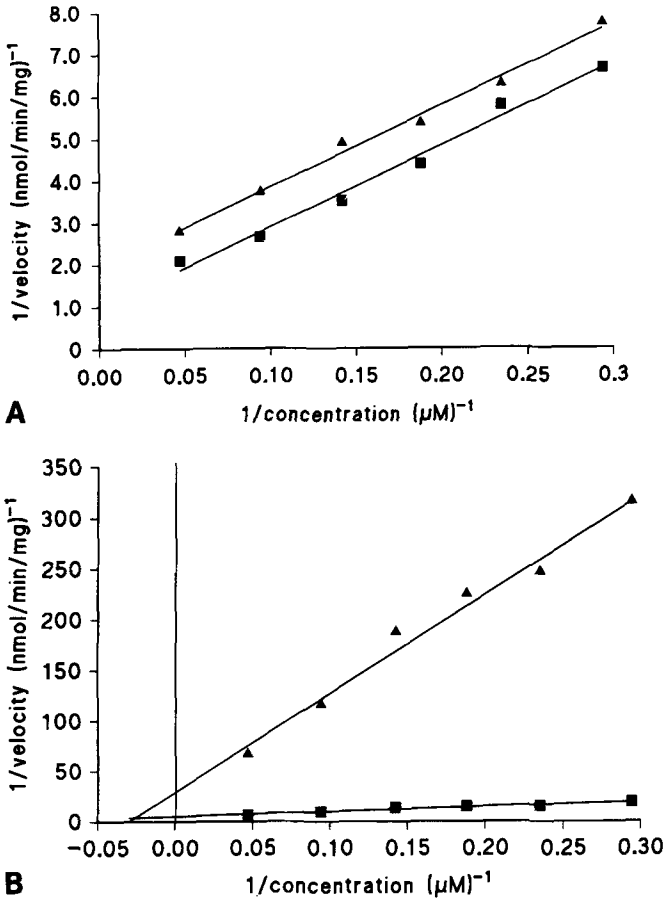

FIG. 3. Inhibitory effect of the cytosolic fraction on neutral CEH in rat mammary gland microsomes (100 μg). Lineweaver-Burk plots of cholesteryl ester hydrolysis in the presence (▲) or absence (■) of the cytosolic protein (50 μg). (a) Reaction initiated by the addition of an ethanolic solution of cholesteryl oleate. (b) Reaction initiated by the addition of rat mammary gland microsomal membranes suspended in an aqueous solution of BSA as detailed in Materials and Methods. The microsomal enzyme activity (pmol cholesteryl ester hydrolyzed per min per mg protein) was 484.8 ± 33.4 in (a) and 159.2 ± 3.5 in (b) at a substrate concentration of 21 μM.

Gel filtration. The liver cytosol was fractionated by Sephacryl S-300HR (Pharmacia) gel filtration and the inhibitory activity in the fractions assayed against the neutral CEH present in the mammary microsomes (Fig. 4a).

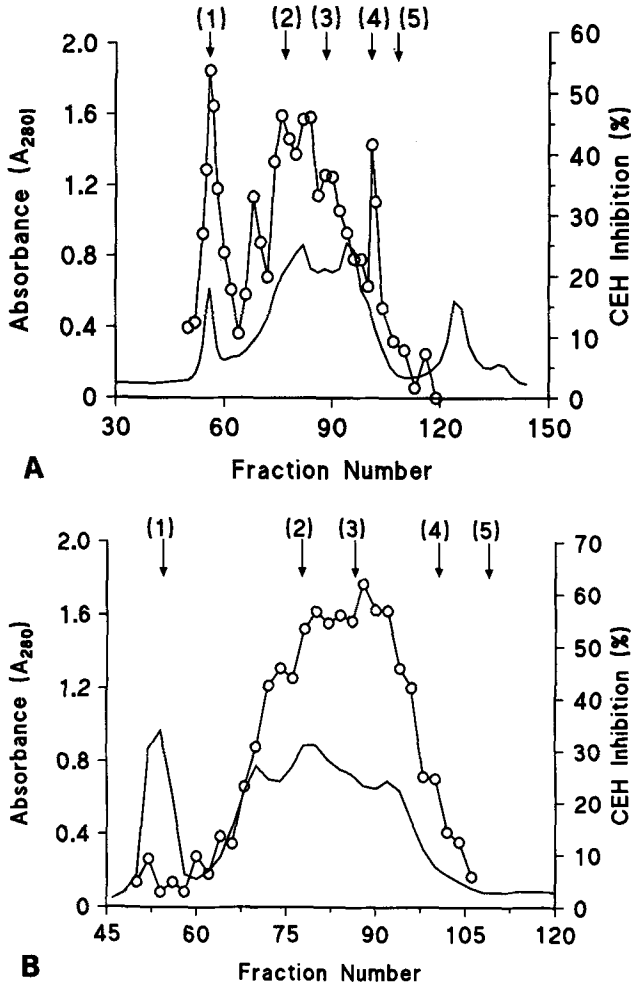

FIG. 4. Gel filtration of (a) liver cytosol (b) extract of acetone/diethyl ether powder on Sephacryl S-300HR. The samples were added to a column of Sephacryl S-300HR equilibrated with 50 mM TRIS, 50 mM MES, pH 7.2, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol at 4°C, and the inhibitory activity in duplicate aliquots of every other fraction determined against the mammary microsomal CEH in the standard assay system, as detailed in Material and Methods. Absorbance at 280 nm (—); inhibitory activity on neutral CEH (-O-). The column was calibrated with the following markers: (1) Blue Dextran 2000; (2) potato amylase (mol wt 200,000); (3) bovine serum albumin (mol wt 66,000); (4) carbonic anhydrase (mol wt 29,000); (5) cytochrome C (mol wt 12,400).

The inhibitory activity eluted over a wide molecular weight range with peaks of activity at M_r 510,000; 220,000; 110,000; and 57,000. A similar multiplicity of peaks was reported by Tanaka and co-workers (22) for a liver cytosolic inhibitor of lysosomal acid CEH. Interestingly, a major peak of activity eluted in the void volume, suggesting the presence of high molecular weight lipoprotein complexes. This interpretation was enhanced when an acetone/diethyl ether powder was prepared from the cytosol and again fractionated on the Sephacryl S-300HR column (Pharmacia). In this instance, very little inhibitory activity eluted in the void volume of the column (Fig. 4b).

Nutritional and diurnal effects. In order to make meaningful comparisons between the different groups of animals, the results are expressed in terms of the amount of cytosolic protein required to achieve 50% inhibition of the neutral CEH in the mammary microsomes under standard assay conditions. The same preparation of mammary microsomal enzyme was employed throughout to prevent possible variations in sensitivity to the inhibitor in different microsomal samples, from obscuring the inhibitory effects of the different liver cytosols. In addition, all of these studies employed extracts of the acetone/diethyl ether powders prepared from the 100,000 × *g* cytosolic fractions of the livers to avoid the possibility that variations in activity could arise from alterations in the hepatic concentrations of cholesteryl esters.

TABLE 5

Inhibition of Neutral CEH in Mammary Microsomes by Hepatic Cytosolic Factors. Influence of Diurnal Variation and Nutritional Status of Animals on Amount of Cytosolic Inhibitor Protein[a]

Animal treatments	Time of sampling	Protein (μg)
Controls	0930	107.20 ± 3.70 (12)
No treatment	1400	97.99 ± 5.52 (5)
No treatment	0200	102.34 ± 4.12
24 h food withdrawal	0930	95.10 ± 5.76
48 h food withdrawal	0930	167.20 ± 7.40*
48 h food withdrawal then 2 h re-feeding	0930	109.32 ± 9.51
Cafeteria feeding	0930	71.20 ± 2.01*

[a]Acetone/diethyl ether powders were prepared from the cytosolic fraction of each of the rat livers as described in Materials and Methods. Extracts of these powders were used as the source of the inhibitory material, and the results are expressed as μg of protein required to achieve 50% inhibition of the neutral CEH activity in mammary gland microsomes. Values are the means ± SEM for six animals, except where indicated by the number in parentheses. Statistically significant differences (Student's *t*-test) between treatments and controls are indicated by $*P < 0.001$.

In the livers of the normally-fed male rats, approximately 100 μg of cytosolic protein was required for 50% inhibition of the neutral CEH in the mammary microsomes (Table 5). Withdrawal of food for 24 h did not alter the inhibitory activity of the liver cytosols, but withholding food for 48 h caused a marked reduction in this activity (indicated by an increase in the amount of protein required to produce 50% inhibition of the microsomal CEH activity). Re-feeding for as little as 2 h restored inhibitory activity to that of the controls. Feeding animals on a high-fat cafeteria-style diet resulted in a decrease in the amount of protein required for 50% inhibition, reflecting an increase in the inhibitory activity of the cytosolic fraction. There was no apparent diurnal variation in the concentration of the inhibitor since the livers of animals taken at the mid-light and mid-dark time points showed essentially the same amount of inhibitory activity which was not different from that exhibited by the control animals sampled 1.5 h into the light phase (Table 5).

DISCUSSION

The concentration of cholesteryl ester in cells is controlled by a balance between synthesis and hydrolysis (32), and a number of disease states are characterized by an accumulation of cholesteryl esters (33-35). Most studies on this problem have concentrated on the synthetic side of this balance, and inhibitors of the enzyme acyl-CoA:cho-

lesterol acyltransferase (ACAT) have been widely employed (36,37). The hydrolytic side of the equation has received less attention, although recent publications have reported studies on a series of synthetic inhibitors of the neutral CEH (38,39). The present work demonstrates that the hepatic cell contains a cytosolic factor(s) that inhibits a number of neutral CEH. Moreover, the higher sensitivity of the liver microsomal CEH to the hepatic cytosolic inhibitor(s) suggests that there may be some degree of tissue specificity in its action. The presence of these naturally occurring inhibitor(s) that can be altered under physiological conditions is potentially of great significance. Moreover, their presence in the cytosol provides an explanation for the reported low activity of hepatic cytosolic neutral CEH (18). The hepatic cytosolic esterase activity *in vivo* could be much higher than that measured *in vitro* due to compartmentalization of the inhibitor and enzyme. This would fit more with the role of the liver as a major site of cholesteryl ester hydrolysis in the rat.

The physiological significance of the inhibitor is also indicated by its variation in response to nutritional changes. Its reduction after 48 h food withdrawal would result in increased hydrolysis of the cholesteryl ester stores reflecting a requirement for maximum utilization of hepatic cholesteryl esters. Cafeteria-feeding (26) would give rise to the opposite situation, leading to maximum storage of cholesteryl esters and, hence, an increased requirement for inhibition of cholesteryl ester hydrolysis. The increase in inhibitory activity within 2 h of re-feeding the starved animal argues against re-synthesis of the inhibitor(s) as a means of increasing its efficiency and implies that other mechanisms control its activity. The activities of neutral forms of CEH in a number of tissues are elevated following phosphorylation by cAMP-dependent protein kinase (16), and analogy with the inhibitors that control the activity of a number of cellular phosphatases (40,41) would suggest modification of the activity of the inhibitor by a similar mechanism. This proposal is currently under investigation.

Interpretation of the mode of action of the inhibitor(s) is complicated by the fact that the substrate is insoluble in aqueous medium and the enzyme would consequently act at a lipid-water interface. The activity of the neutral CEH can be varied by the manner of presentation of the substrate (20,31); the difficulties of assessing inhibitory activities under these circumstances have been recently discussed (42). Nevertheless, consideration of the kinetic parameters reported here suggests certain possibilities. The parallel reduction in K_m and V_{max}, observed when both enzyme and inhibitor were present at the start of the reaction, is stated to be characteristic of an "uncompetitive" form of inhibition (31). The decrease in V_{max} indicates that the activity of the enzyme present in the mixture had been decreased. This could arise from binding of the inhibitor to the enzyme-substrate complex leading to a decrease in its rate of breakdown to free enzyme and products (31). The observed proportionate reduction in K_m is in agreement with this interpretation.

Injection of an ethanolic solution of cholesteryl oleate into aqueous buffer results in the formation of a fine suspension and the hydrophobic nature of the inhibitor, indicated by its binding to phenyl sepharose (Fig. 2), would allow it to form a soluble complex with this suspended substrate (Table 3). When microsomes were then added to initiate the reaction, competition occurred between the free and the bound substrate for the binding sites on the enzyme, leading to an increase in the value for K_m. However, the simultaneous reduction in V_{max} indicates that the resultant inhibition is not of a purely "competitive" type, but is modified by an element of the "uncompetitive" inhibition observed in the previous situation. The bulk of these studies have used a mammary microsomal preparation which is identical to the hormone-sensitive lipase/neutral cholesteryl esterase present in other tissues (14). The inhibitory properties are thus likely to have wider implications than just as a control mechanism in the liver, and we have preliminary evidence that similar inhibitory properties are associated with cytosolic factors from a number of macrophage cell lines. The development of these cells into the foam cells of atherosclerotic lesions by variations in the activity of the inhibitor is an intriguing possibility.

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The Inhibition of Neutral Cholesteryl Ester Hydrolase by a Cytosolic Protein Factor in Female Rat Liver: The Influence of Varying Hormonal and Nutritional Conditions on the Inhibitory Activity

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A cytosolic protein, that is inhibitory to neutral cholesteryl ester hydrolase, has been investigated in the livers of female rats using microsomes isolated from the mammary gland of lactating rats as an enzyme source. To facilitate comparisons, inhibitory activity is expressed in terms of the amount (μg) of cytosolic protein required to reduce esterase activity by 50% and is compared to the hepatic content of both cholesterol and cholesteryl esters. The experiments revealed a sexual difference in the level of inhibitory activity, with the livers of both suckling and mature male animals containing less of the material than the corresponding females. Alterations in the physiological status of the females, such as pregnancy and lactation, led to a decrease in the activity of the protein. This was reversed by blocking lactation with a combination of an antiserum to rat growth hormone and the anti-prolactin drug, bromocriptine, but not by premature weaning of the animals. Food withdrawal for 24 hr also had the effect of increasing inhibitory activity. In general the cholesteryl ester content of the livers correlated with the level of inhibitory activity. Thus the activity of the cytosolic inhibitor of neutral cholesteryl ester hydrolase responded to changes in both the hormonal and the nutritional status of the female animal. It is suggested that the presence of the greater cholesteryl ester hydrolase inhibitory activity in the female liver may help to explain the lower risk of coronary heart disease in premenopausal females by facilitating increased hepatic storage of the sterol in the form of the ester.
Lipids 27, 413–417 (1992).

Hepatic cholesteryl esters, which derive primarily from the uptake and degradation of plasma lipoproteins, are hydrolyzed in liver cells through the action of the lysosomal enzyme cholesteryl ester hydrolase (CEH) having optimal activity at acid pH (1–3). The resulting free cholesterol, as well as that endogenously synthesized, can be catabolized, re-esterified for storage in cytoplasmic lipid droplets, or secreted from the liver in lipoprotein particles. Although the hydrolysis of the plasma-derived lipoprotein cholesteryl ester is mediated by the lysosomal esterase, the cholesteryl ester in the lipid droplets is hydrolyzed by a neutral CEH located in the cytosolic compartment. Free and esterified cholesterol are subject to constant turnover (4) and the neutral CEH thus plays an important role in controlling the supply of free sterol from its storage form. There is considerable evidence that the activity of this neutral CEH in a number of tissues is under hormonal control mediated by a covalent phosphorylation of the enzyme catalyzed by cyclic AMP-dependent protein kinase (5–7). In addition, we have recently described a protein factor in hepatic tissue that inhibits the activity of the neutral cholesteryl esterase (8). Since the activity of the inhibitory material varies under different nutritional and hormonal conditions, this provides a second mechanism for controlling the breakdown of cholesteryl esters and, hence, the output of hepatic cholesterol into the circulation.

Since high levels of plasma cholesterol are one of the factors associated with coronary heart disease, understanding the mechanism by which the activity of this inhibitory material is controlled may help to provide insight into the mechanisms which control plasma cholesterol levels.

Although these original experiments were performed with hepatic tissue from male rats, there is substantial documentary evidence that the hepatic metabolism of cholesterol differs considerably between the male and the female of the species. The two female hormones, estradiol and progesterone, have been reported to modulate cholesteryl ester hydrolysis in the rat (9–13), although the biochemical basis underlying this regulatory effect is not understood. It has, nevertheless, led to the suggestion that the lower risk of coronary heart disease in premenopausal females, in comparison with males of an equivalent age, is due to the presence of estrogens (14). We have therefore extended our investigations to female rats and here show that the livers of these animals do contain the inhibitory material, but in a more active form than that present in the livers of equivalent male rats. This may provide some explanation for the lower plasma cholesterol in females. Furthermore, in confirmation of our results with the male rats, we demonstrate that manipulation of the nutritional and hormonal status of the female rats also produces changes in the activity of the hepatic inhibitor and, hence, alters hepatic cholesterol metabolism.

MATERIALS AND METHODS

Materials. [1-^{14}C]Oleic acid and [9,10(n)-^{3}H]oleic acid were purchased from Amersham International (Amersham, Bucks, U.K.). Cholesteryl [9,10(n)-^{3}H]oleate was synthesized from cholesterol and [9,10(n)-^{3}H]oleic acid as previously described (8), and had a specific activity of (42 mCi/mmol). All biochemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) or from BCL (Lewes, Sussex, U.K.).

Preparation of the inhibitory factor from rat liver. Wistar rats were maintained in an alternating light cycle of 12 hr (light from 0800) and were fed *ad libitum* on a CRM(X) breeding diet (Special Diet Services, Manea, Cambridgeshire, U.K.). Female rats were killed at two stages of pregnancy (day 12 and day 20) and in early lactation (day 2) and mid-lactation (day 10–14). Animals which had failed to mate were used as controls for the pregnant and lactating animals. Animals at mid-lactation also were treated with bromocriptine (0.5 mg) or anti-rat growth hormone (220 mg), or a combination of the two given as two injections per day (at 0900 and 1700 h) for

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; CEH, cholesteryl ester hydrolase; LPL, lipoprotein lipase; VLDL, very low density lipoprotein.

two successive days. Control animals were given injection carrier alone. Where indicated, rats were deprived of food for 24 h. Animals were killed by cervical dislocation at 0930 and the livers excised and frozen in liquid nitrogen. They were homogenized and the acetone/diethyl ether powders prepared from the 100,000 × *g* supernatants of the homogenates as described (8).

Preparation of mammary microsomes. Microsomes were prepared from the mammary glands of rats in mid-lactation as previously described (15) and were stored at −80°C as a suspension in the medium used for the homogenization of the livers.

Assay of CEH. Neutral CEH activity in the microsomal membranes (100 μg) was routinely assayed in the presence and absence of the inhibitory fraction (40–120 μg) as described previously (8).

Protein measurement. The protein concentrations were determined by the dye-binding technique of Bradford (16) using crystalline bovine serum albumin as standard.

RESULTS

In order to make meaningful comparisons beween the different groups of animals, all of the assays were carried out using the same batch of mammary microsomal membranes. The results are then expressed in terms of the amount of cytosolic protein calculated to be necessary to achieve 50% inhibition of the neutral CEH in the mammary microsomes, under standard assay conditions. Thus, a smaller figure represents an increase in the amount of the inhibitory material present in the livers of the experimental animals. Moreover, since we have demonstrated previously that the activity of the inhibitory material was not affected by preparation of an acetone/diethyl ether powder from the liver cytosols, this methodology was adopted here. This has the advantage that residual cholesteryl esters were removed from the cytosol, and any variation in the hepatic content of this lipid could not affect the specific activity of the cholesteryl oleate used in the esterase assay. Furthermore, residual estradiol and progesterone also were removed to nullify the reported direct inhibitory effects of these hormones (10).

In comparison with the females, 35.6% more of the male hepatic cytosolic protein was required to achieve 50% inhibition of the mammary microsomal CEH (Table 1). The results indicate that the inhibitor in the livers of male rats was less potent than that in the female rats. This variation in inhibitory properties of the cytosols of adult male and female livers did not lead to significant differences in the levels of hepatic free and esterified cholesterol (Table 2). There was no evidence of an age-related variation in inhibitory activity in adult animals since the livers of young female rats (170–190 g) exhibited essentially the same activity as the livers of mature animals (280–320 g) (Table 1). However, the inhibitory activity in the livers of the 13-day-old suckling rats was significantly higher than that in the livers of adult animals. The differential noted between adult male and adult female animals was emphasized in these sucklings, where 42.8% more male cytosolic protein was required to achieve the same inhibition as that obtained with the females.

Effect of pregnancy and lactation. Since it is well established that female hormones have a marked effect on hepatic cholesterol metabolism, the effect of pregnancy and lactation on the activity of the hepatic inhibitory material was investigated. The effectiveness of the inhibitory activity showed a steady decline throughout pregnancy and lactation (Table 2). However, it was only in the livers of the animals at mid-lactation (day 10–14) that the differences from the mature controls achieved significance. In comparison with the controls, the mid-lactating animals required 31% more of the cytosolic protein to achieve 50% inhibition of the CEH activity in the

TABLE 1

The Influence of Sex and Age on the Activity of the Hepatic Inhibitor of Neutral CEH[a]

Animals	Age/weight	Inhibitor activity (μg of protein for 50% inhibition)
Adult males	300–340 g	107.20 ± 3.70(12)
Adult females	170–190 g	79.04 ± 1.62(5)[c]
Adult females	280–320 g	79.31 ± 3.82(4)[c]
Suckling males	13 days	81.80 ± 2.14(3)[e]
Suckling females	13 days	57.22 ± 4.76(3)[b,d]

[a]The results are the means ± SEM for the number of animals shown in parentheses and are expressed in terms of the amount of cytosolic protein required to accomplish a 50% inhibition of the mammary gland neutral CEH under standard assay conditions. Significant differences (Student's t-test) between males and females are indicated by [b], $P > 0.01$; [c], $P > 0.001$; and between adults and sucklings by [d], $P > 0.01$; and [e], $P > 0.001$.

TABLE 2

The Influence of Pregnancy, Lactation and Premature Weaning on the Hepatic Content of Free and Esterified Cholesterol and on the Activity of the Hepatic Inhibitor of Neutral CEH[a]

Condition	Inhibitory activity (μg protein for 50% inhibition)	Cholesterol (mg/100 g of tissue)	Cholesteryl (mg/100 g of tissue)
Virgins (av. 180 g)	79.04 ± 1.62(5)	151.3 ± 5.4	88.0 ± 7.0
Virgins (av. 300 g)	79.31 ± 3.82	n.d.	n.d.
Pregnant (day 12)	85.84 ± 3.67	159.8 ± 2.8	81.0 ± 1.2
Pregnant (day 20)	88.10 ± 2.79	138.3 ± 1.5	74.3 ± 0.5
Lactation (day 2)	92.41 ± 5.50	148.3 ± 4.1	81.5 ± 3.0
Lactation (day 12)	103.64 ± 3.04(15)[c]	173.8 ± 2.7[b]	95.3 ± 1.6
Litter-removed	104.92 ± 4.28(9)[c]	125.5 ± 4.3[b,e]	84.0 ± 2.4[d]
Male rats	107.20 ± 3.70(12)	166.8 ± 4.2	74.0 ± 5.0

[a]Litter-removed were without pups for 48 h prior to sampling. The results are the means ± SEM for four animals except where indicated by the numbers given in parenthesis. Inhibitory activity is expressed in terms of the amount of cytosolic protein required for 50% inhibition of the mammary CEH under standard conditions, n.d., not determined. Statistical differences (Student's *t*-test) are relative to the virgin animals [b], $P > 0.01$; [c], $P > 0.001$; between mid-lactators and litter-removed; [d], $P > 0.01$; [e], $P > 0.001$. The results for male animals are included for comparison.

TABLE 3

The Influence of Short-Term Starvation and Insulin Deprivation on the Hepatic Content of Free and Esterified Cholesterol and on the Activity of the Hepatic Inhibitor of Neutral Cholesterol Ester Hydrolase[a]

Condition	Inhibitory activity (μg protein for 50% inhibition)	Cholesterol (mg/100 g of tissue)	Cholesteryl ester (mg/100 g of tissue)
Pregnant (day 20) (24 h starved)	74.99 ± 1.95[d]	189.3 ± 6.5[e]	100.3 ± 2.7[e]
Lactation (day 12) (24 h starved)	85.09 ± 3.44(3)[e]	166.0 ± 3.3	87.8 ± 1.9
Streptozotocin	121.64 ± 3.58[c]	186.8 ± 4.5[b]	104.8 ± 4.7

[a]The starved animals had food withdrawn for 24 h prior to sampling. Streptozotocin (65 mg/Kg) was administered by intraperitoneal injection and the livers taken 3 h later. The results are the means ± SEM for four animals at each condition, except where indicated in parenthesis. Statistical differences (Student's *t*-test) expressed relative to the virgin females are indicated by [b], $P > 0.01$; [c], $P > 0.001$; expressed relative to the appropriate non-starved animals are indicated by [d], $P > 0.01$; [e], $P > 0.001$.

microsomes. The level of inhibitory activity in these mid-lactating animals was therefore similar to that seen in adult males. This was reflected in increases in the amount of free cholesterol present in the livers of the animals in mid-lactation although the cholesteryl ester content was not significantly altered.

Short-term starvation (24 h) of the late pregnant animals resulted in significant increases in the inhibitory activity (Table 3), to levels closer to those seen in the virgin animals. This was correlated with a highly significant increase in the hepatic cholesterol and cholesteryl ester content. Similar treatment (24-h starvation) of the mid-lactating animals also increased inhibitory activity but, in these animals, only a small decrease in cholesterol and cholesteryl ester content was observed. For comparison, starvation of male rats for 24 h had no effect on the hepatic inhibitory activity (8).

Influence of insulin. Lactating animals are hypoinsulinemic (17) suggesting that insulin may have an effect on the inhibitory activity. Short-term hypoinsulinemia has been induced in experimental animals following an injection of streptozotocin (17–19), and this technique was employed to investigate the influence of insulin on the inhibitory activity. Three hours after an injection of streptozotocin, the activity of the cytosolic inhibitor was significantly reduced with 69% more cytosolic protein being required to achieve 50% inhibition of the mammary microsomal esterase (Table 3). Furthermore, the drug significantly increased the hepatic concentration of free cholesterol and also appeared to increase cholesteryl ester levels. In contrast, short term (1 h) injections of insulin and glucose into mid-lactating animals did not alter inhibitory activity (results not shown). Thus, although reductions in insulin plasma levels do apparently influence the activity of the inhibitory material, other factors are also important.

Effect of growth hormone and prolactin. The mammary gland in the lactating rat extracts large amounts of circulating triacylglycerols and cholesterol to provide lipids for milk secretion. Inhibition of this secretory activity, which can be achieved by reducing the plasma levels of growth hormone and/or prolactin, would be expected to have marked effects on hepatic lipid metabolism. Rats in mid-lactation were therefore treated for two days, either with an antiserum to growth hormone or with the anti-prolactin drug, bromocriptine, or with a combination of the two agents. Although both bromocriptine and the antiserum individually produced a reduction in milk yield (20), neither was able to promote any alteration in the activity of the inhibitory material in the livers (Table 4). However, when the two agents were administered simultaneously, a 29% increase in the activity of the inhibitory material was observed. This brought it to the level that was found in the livers of non-lactating animals. The complete cessation of milk production in the mammary gland, produced by the simultaneous injection of these two anti-hormone agents, is similar to that obtained when the animals are prematurely weaned (21). However, removal of the litters for 48 h prior to sampling made no difference to the effectiveness of the inhibitory material in the liver, which remained at the same level as that found in the mid-lactating animals (Table 2). Although the two anti-hormone agents did not change inhibitor activity when administered separately, individually they caused significant decreases in the hepatic level of both cholesterol and cholesteryl ester. However, with the combined administration of the antiserum and bromocriptine, hepatic cholesterol levels were unaltered from controls, whereas cholesteryl ester levels were increased, as was consistent with the enhanced activity of the inhibitor.

DISCUSSION

The results presented here indicate that the hepatic inhibitor of neutral CEH, identified in a previous report (8), is more active in the female liver than in the male liver. Sexual differences in rat hepatic cholesterol metabolism have been attributed to the action of the female sex hormones estradiol and progesterone (9,14,22–26). Administration of ethinylestradiol to rats results in an increase in hepatic esterified cholesterol and an apparent decrease in CEH activity (9,10,26). The increased inhibitory activity seen in the female livers would therefore correlate with this effect of the estrogen and may help

TABLE 4

The Activity of the Inhibitory Cytosolic Protein Factor in the Livers of Rats in Mid-Lactation: Influence of Variation in Serum Prolactin and Growth Hormone Levels[a]

Treatment	Inhibitory activity (μg protein for 50% inhibition)	Cholesterol (mg/100 g of tissue)	Cholesteryl ester (mg/100 g of tissue)
Control	103.64 ± 3.04(15)	173.8 ± 2.7	95.3 ± 1.6
Antiserum to growth hormone	106.50 ± 12.60(5)	152.5 ± 6.7[b]	76.0 ± 4.7[c]
Bromocriptine	107.70 ± 9.40(6)	156.0 ± 2.7[c]	82.8 ± 2.1[c]
Antiserum + bromocriptine	80.30 ± 4.20(11)[c]	181.5 ± 3.1	104.3 ± 3.3[b]

[a]Rats in mid-lactation were treated with the various combinations of agents and the livers analyzed for inhibitor activity and cholesterol and cholesteryl ester content as described in Materials and Methods. The results are the means ± SEM for four animals except where indicated by the numbers in parenthesis. Inhibitory activity is expressed in terms of the amount of cytosolic protein required to achieve 50% inhibition of the mammary microsomal CEH under standard conditions. Significant differences, analyzed by Student's *t*-test, are indicated between treatments and controls: [b], $P > 0.05$; and [c], $P > 0.002$.

to explain some of the well documented differences in cholesterol metabolism between the sexes. In spite of the difference in inhibitor activity between males and females, there was no significant difference in their hepatic cholesteryl ester contents. This may reflect the fact that these were untreated animals, whereas the reported increases in cholesteryl ester content resulted from treatment with pharmacological doses of the estrogens (26). Alternatively, it may reflect the presence of separate pools of free and esterified cholesterol (27–34), only some of which are influenced by the inhibitor.

Progesterone increases the biliary output of cholesterol (35) and inhibits the activity of acyl-CoA:cholesterol acyltransferase (ACAT), the enzyme responsible for cholesterol esterification (36,37). High progesterone levels occur in late pregnancy, so the observed decrease in hepatic cholesterol content is consistent with the proposition of increased biliary output of the sterol. Replenishment of the hepatic cholesterol pool may involve an increase in CEH activity, a suggestion supported both by the decrease in the hepatic cholesteryl ester content and by the reduction in inhibitor activity. It is interesting to note that young women taking oral contraceptives (high in progesterone) are more susceptible to gallstones (38), raising the possibility that variations in the activity of this inhibitor is a factor in the etiology of this disease.

The results obtained with pregnant animals following 24-h starvation are also consistent. The reduction in biliary output would increase the hepatic cholesterol content and provide more substrate for ACAT, leading to a greater cholesteryl ester content. The effect of a higher inhibitory activity on the esterase would ensure that this increased level of cholesteryl ester was maintained.

Hepatic cholesterol synthesis is increased in lactation (39), but the hyperphagia associated with this physiological state (17) imposes an additional requirement for cholesterol for increased secretion of very low density lipoproteins (VLDL) (40,41). This correlated with the decreased inhibitory activity in these animals which would permit increased hydrolytic breakdown of the stored cholesteryl esters. Following short-term starvation (24 h), VLDL secretion would be diminished and hepatic storage of the esterified sterol would assume a greater importance. Thus, inhibitory activity was increased in response.

Bromocriptine has been reported to decrease mammary lipoprotein lipase (LPL) activity, but not the accumulation of lipid by the mammary gland (42), indicating that continued secretion of hepatic VLDL was required. Therefore, it was not surprising that the level of hepatic inhibitory activity in the lactating animals was unaltered when this drug was administered. More paradoxical was the lack of change in inhibitory activity following removal of the pups, since this was reported to cause a massive decrease in lipid accumulation in the mammary gland (42). However, in weaned animals, the LPL activity of white adipose tissue was increased and lipid deposition in the adipose tissue became predominant (42). The level of inhibitory activity in the liver may thus reflect a continued demand for cholesterol, for VLDL secretion, and this correlates with the reduction in free and esterified cholesterol in the liver.

The effects of injecting bromocriptine together with the antiserum were more puzzling. This treatment was reported to reduce milk yield (20) and to both reduce mammary LPL activity and elevate adipose tissue LPL to a level similar to that obtained on litter removal (Clegg, R.A., unpublished data). In the current experiments an increase in hepatic cholesteryl ester was observed concomitant with a decrease in inhibitor activity. No explanation for this difference between the litter removal and the anti-hormone treatments can be provided, although it should be noted that the two physiological conditions are not identical. The pups continued to suckle during the anti-hormone treatment and the mammary gland was depleted of milk, whereas weaning led to an accumulation of milk. Alternatively, since the liver has specific receptors for both prolactin and growth hormone, the observed result may arise from a direct effect on the liver, due to

removal of these hormones, rather than from a secondary effect related to the hepatic secretion of cholesterol.

The differences in inhibitor activity in the suckling animals are also interesting since sex differences in cholesterol metabolism normally only become apparent after puberty at about 6-7 weeks of age (43). Suckling animals, nevertheless, do show large sexual differences in the hepatic enzymes that metabolize steroids (25,44-46). Thus, the observed variations in the inhibitory activity in these animals suggests that the inhibitor is of fundamental importance, and may function not only in the control of hepatic cholesterol metabolism, but also possibly in controlling other aspects of steroid metabolism. Finally, the lower risk of coronary heart disease in premenopausal women when compared with males of equivalent age has been repeatedly confirmed by clinical and epidemiologic studies. The evidence that the female liver contains a more highly active inhibitor of the hepatic CEH, leading to increased hepatic storage of cholesterol as the ester, may help to provide an explanation for some of these differences. If this were proven then pharmacological manipulation of this inhibitory activity may constitute an important advance in the treatment of a number of diseases characterized by altered cholesterol metabolism.

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Cholestane as a Digestibility Marker in the Absorption of Polyunsaturated Fatty Acid Ethyl Esters in Atlantic Salmon[1]

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Salmonid fish require long-chain n-3 fatty acids in their diet. The digestibility of different chemical forms of fish oil fatty acids, fed as triacylglycerols, free fatty acids or ethyl esters, was examined in 300 g farmed Atlantic salmon (*Salmo salar*) using cholestane as an indicator of fat absorption *in lieu* of the chromium oxide (Cr_2O_3) which is commonly used as a marker in digestibility studies. It was established that the two digestibility markers gave similar results. Conveniently, cholestane does not require a separate analysis if fatty acids are to be determined by appropriate gas-liquid chromatography. The long-chain polyunsaturated fatty acids were particularly well absorbed, the apparent digestibility being 90–98% when feeding triacylglycerols or free fatty acids. However, the digestibility of monounsaturated fatty acids (75–94%) was lower, and lower still for saturated fatty acids (50–80%). Ethyl esters of fatty acids were significantly less well absorbed ($P < 0.05$) than were the corresponding fatty acids in free acid or triacylglycerol form. Irrespective of dietary fat type, only free fatty acids were identified in feces, indicating total hydrolysis of triacylglycerols and ethyl esters.
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Ethyl esters or free fatty acids are the most economical forms in which highly concentrated marine n-3 fatty acids are available in bulk (1,2). Some researchers have assumed that ethyl esters of n-3 fatty acids are efficiently absorbed by humans and rats (3–5). As ethyl esters are not a natural food, it is possible that they are not hydrolyzed by lipases as efficiently as are natural fats in humans and in animals (6–8). On the basis of previous *in vitro* work (9) it has been assumed that long-chain n-3 polyunsaturated fatty acid esters with ethylenic bonds near the carboxyl group may be resistant to pancreatic lipolysis in mammals. This may reduce absorption and lower transfer of these fatty acids to lymph and blood (10). However, others have suggested that once triacylglycerols containing n-3 fatty acids are hydrolyzed, the n-3 fatty acids liberated are totally absorbed *in vivo* (6,7,11). Indeed, since the digestion *in vivo* may go on for hours, the rate of lipolysis *in vitro* may have little influence on the total amount of fat absorbed.

Polvi (12) observed that ethyl esters were absorbed by Atlantic salmon, although the absorption efficiency was not determined. She also noted that growth was less than with triacylglycerol oils. It has been established that the physiological specificity of hydrolysis by fish intestinal juices is markedly different from that of human pancreatic lipase, for example (13,14). The products of intraluminal hydrolysis of triacylglycerols in mammals are monoacylglycerols and fatty acids. In salmonids, monoacylglycerols are, to a large extent, hydrolyzed into fatty acids and glycerol (13). Recent work with cod (*Gadus morhua*) has isolated a bile salt dependent lipase capable of 1,3-hydrolysis of trioleoylglycerol, and also capable of hydrolyzing methyl esters of fatty acids (15). However, another enzyme, a nonspecific lipase different from pancreatic lipase, that competes effectively with lipase as a major fat digestive enzyme in fish, may be involved (13,14). An additional example of hydrolysis is that of wax esters, important food components for clupeid fish (16,17), but also digested by cod (18). If more than one lipase is present in the gastrointestinal tract of fish (13) there exists the possibility of equally rapid and complete absorption of fatty acids fed as triacylglycerols or as ethyl esters. Although the digestibility of dietary lipids in triacylglycerol form has been discussed (19), there are no reports on the absorption of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) when fed to fish in either ethyl ester or free fatty acid form.

In digestibility studies, inert markers or indicators such as chromium oxide (Cr_2O_3) are commonly used (20). However, Cr_2O_3 is not without some problems (21), and has one serious disadvantage in that it has to be determined by an analysis separate from fat or fatty acids. In lipid nutrition, a marker preferably should be recovered along with lipids, and also be determined readily by lipid analytical techniques. The hydrocarbon squalene has been examined as a digestibility marker for absorption of other hydrocarbons in rats by Albro and Fishbein (22). It is established that, in general, the extent to which alkanes are absorbed by mammals decreases with chain length and with increasing melting point (22,23). Unfortunately, alkanes are often present in both plant and animal food sources (24), but cholestane appeared to be a xenobiotic and offered a suitable set of physical and chemical properties for this role.

The objectives of the present study were to investigate cholestane as a suitable marker for lipid digestibility studies with fish, and to determine the absorption of ethyl esters of fatty acids and especially of the polyunsaturated fatty acids of marine oils.

MATERIALS AND METHODS

Fish and diets. Two experiments were conducted. The first experiment was designed to examine the suitability of cholestane as an indicator of fat absorption, the second to apply this marker in a digestibility study. In both studies, Atlantic salmon smolts (averaging 300 g each) were acclimated to feed, aquaria and standardized experimental conditions (25). Seawater was supplied to each tank at a flow rate of 3 L per min and maintained at 15 ± 1°C in a flow-through system. The photoperiod (12

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EE, ethyl ester; FFA, free fatty acid; GC, gas chromatography; TLC/FID, thin-layer chromatography with flame ionization detection.

h daily) was controlled automatically. Feeding to satiety was by hand (twice) during the lighted period.

In a preliminary study, a commercial diet containing 16% fat was supplemented with herring oil containing cholestane (Sigma, St. Louis, MO), to increase the fat level of the diet to 22% and to provide cholestane at 0.25% of diet lipid. After three days of feeding, fish were anaesthetized by immersion in seawater containing MS 222 (ethyl *m*-aminobenzoate methanesulfonate). Blood samples (1 mL) were drawn from the caudal artery in a heparinized syringe, and feces were collected from the rectal region of three fish by applying gentle pressure to the surface of the fish from the ventral fins towards the anus.

In the main study, a casein-gelatin based purified diet (Table 1) was used to determine the digestibility of marine oil fatty acids which were supplied in three different chemical forms in the diet. Twenty-five Atlantic salmon smolts per tank in each of five tanks were fed five separate test diets (Table 1) with four of the diets differing only in lipid source (15% of diet). The following four lipid supplements were tested: 15% Menhaden oil (Diet 1); 15% free fatty acids of menhaden oil (Diet 2); 15% ethyl esters of menhaden oil (Diet 3); and 15% of a mixture (1:1) of ethyl esters of menhaden oil and of corn oil (Diet 4). Cholestane was dissolved in the various lipid supplements (before they were mixed with the basal diet) to give a level of 0.7 mg/g diet (equal to 0.5% of fatty acids). The fifth diet, with 15% menhaden oil, was prepared by incorporating chromic oxide (Fisher Scientific Ltd., Fairlawn, NJ) at a level of 0.5% as a standard digestibility marker instead of cholestane. The first five days constituted an acclimation period and no feces were collected during this period. After five days fish were anaesthetized and feces were collected. Feces were stripped from respective groups of approximately 12 fish at one time (a.m. and p.m. subsamples of approximately 5 g each were obtained from each tank on each day and pooled). This procedure was repeated on the same fish after three days. Feces samples were immediately frozen at −30°C until required for analysis.

Preparation of ethyl esters, free fatty acids and analysis of diets. The ethyl esters and free fatty acids were made from menhaden oil according to Christie (26), and Nadenicek and Privett (27), respectively, with minor modification. An Iatroscan TH-10 Mark III TLC/FID (thin-layer chromatography with flame ionization detection; Newman-Howells Associates Ltd., Penybont Uchaf, Great Britain) was used to determine the chemical purity of the ethyl esters and free fatty acids prepared from menhaden oil. The procedure was basically that described by Parrish and Ackman (28). The Iatroscan also was used for quantification of lipid classes in feces. Area measurements were performed with a SP4200 computing integrator. The air flow was 2 L/min and the detector hydrogen flow rate 160 mL/min. The recording chart speed was 16 cm/min and the scanning speed 0.42 cm/s. The yield of ethyl esters and free fatty acids was 85%, and the chemical purities 99% and 89%, respectively.

Lipid and fatty acid analysis. Duplicate samples of each experimental diet (20 g) were weighed, placed in a beaker with 70 mL of water and sonicated for 15 min. The solution was then mixed in a Waring Blendor for chloroform/methanol extraction of lipid by the method of Bligh and Dyer (29). Lipids were extracted from feces and plasma by the Folch method as modified by Christie (26, and references therein). Dilute hydrochloric acid solution (3N) was added to the last extraction of the feces, to destroy any calcium soap present. Total lipids were examined for lipid classes by Chromarod-Iatroscan (only μg being required), with development in hexane/diethyl ether/formic acid (97:3:1, vol/vol/vol). The recovered lipids were transesterified and/or esterified by the method of Morrison and Smith (30). Recovered methyl esters of fatty acids and the cholestane were determined simultaneously by capillary gas chromatography (GC) in a Perkin-Elmer model 900 gas chromatograph (Perkin Elmer, Norwalk, CT) fitted with a SUPELCOWAX-10 (bonded polyethylene glycol) fused silica column, dimension 30 m × 0.32 mm, phase thickness 0.2 μm (Supelco Canada Ltd., Oakville, Ontario, Canada). Helium pressure was set at 76 kPa. The temperature program used was isothermal at 195°C for 8 min, followed by an increase at 3°C/min to 240°C, and a final isothermal hold of 20 min. For the determination of fatty acid composition the weight percentage of each fatty acid was calculated according to Ackman and Eaton (31). Peaks were identified by comparing the retention times with those of standard mixtures of known fatty acid methyl esters. Cholestane was used to calculate digestibility by the equation:

$$D(\%) = 100 - \frac{\text{(area chol-feed)}}{\text{(area chol-feces)}} \times \frac{\text{(area fa-feces)}}{\text{(area fa-feed)}} \times 100$$

where areas are obtained from peaks of GC chromatograms (chol-feed, cholestane in feed; chol-feces, cholestane in feces; fa-feed, fatty acid in feed; fa-feces, fatty acid in feces).

TABLE 1

Composition of the Experimental Diets in the Exploratory Phase

Feed ingredients	Amount (w/w %)
Casein	40.0
Gelatin	8.0
Dextrin	9.5
Starch	8.3
D-glucose	4.0
Carboxymethylcellulose	2.0
Alpha cellulose	5.0
Vitamin mixture[a]	1.0
Mineral mixture[b]	4.0
Amino acid-mixture[c]	3.0
Choline chloride	0.2
Lipid supplement[d]	14.93
Cholestane[e,f]	0.07

[a]Supplied vitamins at following levels: (I.U. or mg/kg) Thiamin, 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 I.U.; vitamin D_3, 2400 I.U.; vitamin E, 300 I.U.; and vitamin K, 40.

[b]Supplied minerals at following levels: (mg/kg) Manganese, 50; iron, 60; zinc, 120; and copper, 25.

[c]Amino acids supplied at following levels (%): DL-methionine, 0.5; L-arginine-HCl, 1.0; and betaine amino acid additive, 1.5 (Finnstim, Finnish Sugar Ltd., Finland).

[d]Lipid supplements had the following forms. Diet 1, menhaden oil; Diet 2, free fatty acids of menhaden oil; Diet 3, ethyl esters of menhaden oil; Diet 4, ethyl esters of menhaden oil and triacylglycerols of corn oil (1:1); and Diet 5, menhaden oil.

[e]Cholestane was dissolved in lipid supplements 1–4 prior to mixing.

[f]Diet 5 contained chromic oxide instead of cholestane.

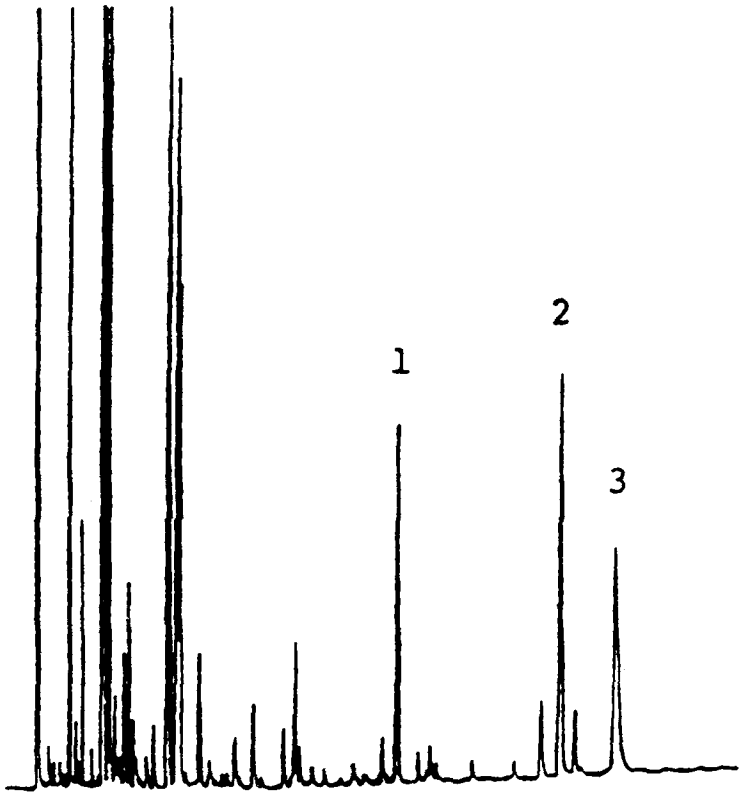

FIG. 1. Gas-liquid chromatogram of methyl esters of fatty acids recovered from feces of Atlantic salmon on a fish oil diet. Peak designations: 1, Eicosapentaenoic acid; 2, docosahexaenoic acid; and 3, cholestane. Other peaks represent saturated, monounsaturated and polyunsaturated fatty acids common in fish oil.

Chromic oxide analysis. Chromic oxide in Diet 5 and in the associated feces was determined according to the method of Arthur (32).

Statistics. Data sets were analyzed by analysis of variance and two-sample *t*-tests. Differences were considered significant if $P < 0.05$. The arcsin transformation (33) was used where statistical comparisons were performed on proportion data. The F-test was used to determine whether the standard deviations of the sample were significantly different ($P < 0.05$).

RESULTS AND DISCUSSION

Cholestane as a digestibility marker. Cholestane ($C_{27}H_{40}$, MW 372.65, m.p. 80°C) was selected on the basis of its physical and chemical properties, such as lipid solubility and the absence of interference with the analysis of fatty acid methyl esters on "bonded" polyglycol wall-coated open-tubular gas chromatography columns (Fig. 1). Cholestane has not been used previously as a digestibility marker. Squalane has been recommended as a lipid digestibility marker in rats (22), but squalane was unsuitable because of the possibility of interference with the analysis of longer-chain fatty acid methyl esters when analyzed by GC on the SUPELCOWAX-10 column. Chromic oxide, the most commonly used digestibility marker (20,21), is not fat soluble and must be determined separately from fat and fatty acids.

In a preliminary study, after feeding diets supplemented with cholestane for three days, cholestane was found to be highly concentrated in the fecal fatty acids, 3.0% *vs.* 0.3% in those of the feed. Cholestane was detected in fish plasma lipids at very low levels (Table 2). To estimate the absorption of cholestane, it was assumed that a dietary lipid steady state was achieved in plasma concentrations of cholestane after continuous feeding of diets *ad libitum* for three (or five) days, and that plasma n-3 polyunsaturated fatty acids were mostly freshly absorbed (34) and represented dietary fat. From this viewpoint, cholestane is calculated to be absorbed at less than 5% of n-3 polyunsaturated fatty acids. The proportion of cholestane absorbed could reflect the various other types of lipids present in the diet. In this context, the results of the preliminary study could be applied with confidence to Diets 1 and 2 based on triacylglycerols. The similarity of results for ethyl esters only and ethyl esters plus triacylglycerols (Table 3) suggests that cholestane was equally suitable for other high-fat diets.

Comparison of cholestane and chromic oxide as digestibility markers. The digestibility of fatty acids in the form of triacylglycerols was not significantly ($P > 0.05$) different when using either chromic oxide or cholestane. However, the standard deviation was observed to be higher for all of the fatty acids except one, and significantly higher ($P < 0.05$) for three fatty acids when using chromic oxide (Table 4). To determine fatty acid absorption with cholestane as a digestibility marker, the proportion of cholestane and fatty acids in the feed could be com-

TABLE 2

Representative Total Fatty Acid Profiles (w/w %) of the Fatty Acids in the Diet, Feces and Plasma of Atlantic Salmon and Cholestane Area Relative to Fatty Acids After Feeding a Commercial Diet Supplemented with Cholestane for Three Days[a]

Fatty acid	Diet (%)	Feces (%)	Plasma (%)
12:0[b]	0.1	0.1	0.1
14:0	7.6	7.0	3.3
15:0	0.7	0.7	0.5
7-me-16:0[c]	0.3	0.2	0.3
16:0	15.2	19.6	19.0
16:1n-7[d]	7.5	3.0	4.6
16:1n-5	0.3	0.2	0.2
16:2n-6	0.2	0.1	0.1
16:2n-4	0.8	0.3	0.6
16:3n-3	0.6	0.1	0.4
16:4n-1	1.0	0.2	0.3
17:0	0.2	0.4	0.3
18:0	1.8	3.6	2.6
18:1n-9	10.5	5.5	11.4
18:1n-7	2.1	1.7	1.9
18:1n-5	0.3	0.3	0.4
18:2n-6	4.4	2.2	3.2
18:2n-4	0.2	0.1	0.06
18:3n-6	0.1	0.1	0.1
18:3n-3	1.0	0.4	0.5
18:4n-3	1.9	0.3	0.9
20:0	0.3	0.5	0.2
20:1n-9	9.2	12.0	5.4
20:1n-7	0.4	0.5	0.4
20:2n-6	0.2	0.2	0.3
20:3n-6	0.1	0.1	0.3
20:4n-6	0.1	0.3	1.3
20:4n-3	0.6	0.2	0.6
20:5n-3	7.0	1.5	10.4
21:5n-3	0.3	0.2	0.3
22:0	0.1	0.1	0.2
22:1n-11 + 13[e]	16.2	28.0	5.5
22:1n-9	1.0	0.4	0.5
22:5n-3	0.8	0.4	1.8
22:6n-3	6.3	6.3	22.0
24:1n-9	0.3	0.2	0.01
Cholestane[f]	0.3	3.0	0.03

[a]Data are the mean of two determinations.
[b]Ratio of carbon atoms to double bonds.
[c]Methyl group on C_7.
[d]Number of carbon atoms from the methyl end of the acyl chain to the nearest double bond.
[e]Isomers not separated.
[f]Hydrocarbon marker was added to the diet at a level of 0.25% of lipid or 0.277% of fatty acids.

pared directly to that in the feces. Therefore, cholestane was accepted as a convenient and suitable indicator of fat absorption.

Fatty acid absorption by Atlantic salmon. The apparent digestibility of total fatty acids (84%), fed either as triacylglycerols or as free fatty acids (Table 4), is in agreement with results from Austreng *et al.* (19), who observed digestibility of total fatty acids in rainbow trout to be 84–90%. The long-chain n-3 polyunsaturated fatty acids were particularly well absorbed, the apparent digestibilities being 90–97% when fed either as triacylglycerols or as free fatty acids (Table 3). The digestibility of monounsaturated fatty acids was 75–94%, depending on the chain length. However, the absorption was lower for saturated fatty acids, diminishing from 70% for 14:0 to 50% for 18:0 (20:0 was present at very low levels in the diet, and was difficult to determine accurately). The poor absorption of saturated fatty acids compared to unsaturated analogs of equivalent chain length (Fig. 2) is in agreement with results in rats observed by Carroll (35), and was recently confirmed by de Schrijver *et al.* (36) for C_{18} fatty acids. Lie and Lambertsen (18) examined gut and intestinal contents and considered that specificity of enzyme is a factor in discrimination among fatty acids absorbed by cod (*Gadus morhua*). The reason for preferential absorption of polyunsaturated fatty acids is not obvious, as the longer and more unsaturated fatty acids have been observed to be more slowly hydrolyzed by pancreatic lipase *in vitro* (9). In rats fed fish oils, however, these fatty acids appear almost immediately (30 min) in the lymph (37), so *in vitro* studies can be unreliable guides.

The absorption of the fatty acids esterified to glycerol was inversely correlated with the melting points of the fatty acids (R = 0.92), where the digestibility decreased with increasing melting points (Fig. 3). This has been reported by other investigators (19,38). Austreng *et al.* (19) examined the digestibility of fatty acids of different melting points in mink and in trout, using chromic oxide as the marker. One possible explanation for the low absorption of saturated fatty acids lies in the calcium present in intestinal fluids. This has been reported to affect the fatty acid absorption. Gacs and Barltrop (39) observed both the solubility of fatty acid calcium soaps and the absorption of calcium to be inversely correlated with chain length and the degree of saturation of the dietary fatty acids. The solubility in intestinal fluid could control the final rate of absorption.

The absorptions of EPA and DHA fed as ethyl esters were significantly less ($P < 0.05$) than the absorption of the triacylglycerols and free fatty acid forms of these same long-chain polyunsaturated fatty acids. This was observed when feeding ethyl esters at the 15% level and also at the 7.5% level (Fig. 4, Table 3). This is in agreement with the conclusions of El Boustani *et al.* (6) and of Lawson and Hughes (7), who observed incorporation of EPA and DHA, fed as ethyl esters, into plasma triacylglycerols in humans, and found that the incorporation was lower than when they were administered as triacylglycerols or as free fatty acids. Such "pulse" feeding is not apt to yield the same results as feeding over extended time periods (36). The superior absorption of the ethyl ester of EPA (Fig. 4) as compared to that of DHA is matched by that for methyl esters exposed to the cod lipase (15) at 37°C, where the activity towards EPA was nearly ten-fold that towards DHA. This appeared to be related to fatty acid chain length rather than to the frequently discussed position of the double bond nearest the carboxyl group (40).

When digestibility was calculated for total fatty acids and lipids, the total fatty acids were not absorbed nearly as well ($P < 0.05$) when fed as ethyl esters as when fed as triacylglycerols or free fatty acids (Table 3). This was emphasized by the significantly ($P < 0.05$) higher dry weight (Table 5) in the feces from fish fed ethyl esters (Diets 3 and 4). The absorptions of fatty acids of triacylglycerols and of actual free fatty acids were not significantly different (Table 3). In rats, free acids were not as well absorbed as the same acids in triacylglycerol form (36).

The apparent digestibilities obtained from the first and second fecal collections were not significantly ($P > 0.05$)

TABLE 3

Apparent Digestibilities in Atlantic Salmon of Fatty Acids in Various Chemical Forms[a,b]

Fatty acid	Diet 1 (%)	Diet 2 (%)	Diet 3 (%)	Diet 4 (%)
14:0	78.3 ± 0.4	69.4 ± 4.0	71.3 ± 4.2	68.1 ± 6.6
16:0	64.6 ± 2.8	64.9 ± 4.1	56.1 ± 2.2	64.1 ± 4.0
18:0	52.3 ± 4.1	47.9 ± 0.6	42.1 ± 0.1	58.6 ± 3.0
20:0	68.9 ± 1.3	62.1 ± 6.2	50.3 ± 1.7	61.4 ± 4.2
16:1n-7	93.5 ± 2.9	94.3 ± 0.6	87.1 ± 3.9	81.2 ± 4.3
18:1n-9	90.5 ± 3.6	91.3 ± 0.5	80.3 ± 5.1	76.1 ± 3.9[b]
18:1n-7	87.5 ± 4.8	88.3 ± 0.7	69.3 ± 2.9	72.2 ± 5.2
20:1n-9	85.4 ± 5.9	85.5 ± 3.7	53.8 ± 3.6[b]	68.9 ± 5.0[b]
20:1n-7	82.1 ± 5.6	74.6 ± 3.5	45.6 ± 2.3[b]	71.2 ± 7.0
18:2n-6	90.8 ± 3.1	90.3 ± 2.2	86.5 ± 2.8	81.7 ± 3.2[b]
18:3n-3	93.7 ± 2.1	95.4 ± 1.3	87.9 + 4.2	86.1 ± 2.1[b]
18:4n-3	97.3 ± 1.6	97.1 ± 2.1	95.8 ± 2.0	93.1 ± 3.3
20:4n-3	95.5 ± 2.5	96.7 ± 2.3	86.1 ± 4.6	80.6 ± 3.6[b]
20:5n-3	96.8 ± 1.9	97.5 ± 0.4	92.3 ± 0.5[b]	92.8 ± 0.8[b]
22:5n-3	92.7 ± 4.0	95.8 ± 2.5	88.9 ± 4.1	89.7 ± 0.9
22:6n-3	91.0 ± 4.8	94.4 ± 0.7	74.6 ± 5.4[b]	81.5 ± 1.6[b]
Total fatty acids	84.4 ± 1.8	84.4 ± 1.3	77.4 ± 2.3[b]	77.5 ± 3.0[b]
Lipids[c]	85.9 ± 2.2	86.2 ± 2.5	77.3 ± 1.9[b]	78.1 ± 1.2[b]

[a]Diet 1, triacylglycerols; Diet 2, free fatty acids; Diet 3, 15% ethyl esters; and Diet 4, 7.5% ethyl esters/7.5% corn oil. Data are the mean ± SD of four determinations of the proportion (%) of the fatty acids, except for the group fed ethyl esters 15% (two determinations).

[b]Significantly ($P < 0.05$) different from triacylglycerols and free fatty acids.

[c]Digestibility of lipids is determined by comparing the proportion of lipid and cholestane content of dry weight feed and feces.

TABLE 4

Comparison of Digestibility (%) in Atlantic Salmon of Fatty Acids When Fed as Triacylglycerols, Determined by Using Cholestane or Chromic Oxide as the Marker[a,b]

Fatty acids	Cholestane (%)	Chromic oxide (%)
14:0	78.3 ± 0.4	77.4 ± 5.1[c]
16:0	64.6 ± 2.8	58.0 ± 8.6[c]
16:1n-7	93.5 ± 2.9	92.4 ± 3.3
18:0	52.3 ± 4.1	46.2 ± 10.6[c]
18:1n-9	90.5 ± 3.6	86.9 ± 3.4
18:2n-6	90.8 ± 3.1	90.7 ± 5.4
18:3n-3	93.7 ± 2.1	92.9 ± 2.4
20:5n-3	96.8 ± 1.9	95.1 ± 2.4
22:6n-3	91.0 ± 4.8	87.5 ± 5.8

[a]Data are the mean ± SD of four determinations.

[b]No significant ($P > 0.05$) difference was observed between the mean of the two groups for any fatty acid.

[c]SD is significantly different ($P < 0.05$) from SD observed when using cholestane.

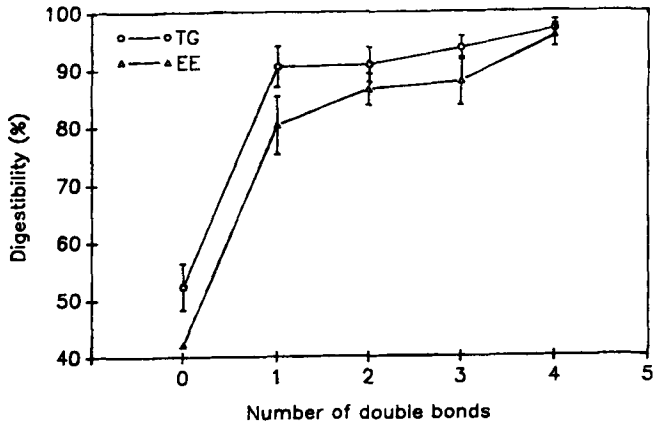

FIG. 2. Digestibility (%) in Atlantic salmon of fatty acids composed of 18 carbons fed as triacylglycerols (TG) and ethyl esters (EE), plotted as a function of their unsaturation. Data are the mean ± SD of four determinations.

different, except for the group fed ethyl esters only (Diet 3). This group had diarrhea at the second collection and the results from this fecal collection were excluded from calculations. The fatty acids in the feces were in the free acid form in all of the groups (Fig. 5), except for the group fed ethyl esters only, at the second time of fecal collection (data excluded). Therefore, all of the dietary lipids were totally hydrolyzed as predicted by Yang *et al.* (40). The diet which contained both corn oil triacylglycerol and marine oil fatty acid ethyl esters (Diet 4) did not show any difference in absorption from the diet which contained the latter as ethyl esters only (Diet 3). This indicates that the added triacylglycerol in Diet 4 did not facilitate the dissolution of the ethyl esters into the intestinal micelles, or otherwise promote digestibility. Such a dissolution step is thus not a rate-limiting factor in absorption. The differences in absorption of fatty acids or ethyl esters could still result from impairment at the ethyl ester hydrolysis step. This was complete (Fig. 5), but could have been completed too late to provide for completion of absorption. The digestive process in fish (13,15,16,18) may not be totally explainable in terms of mammalian pancreatic lipase activity *in vitro* (36,40).

It can be concluded that dietary fatty acids, as ethyl

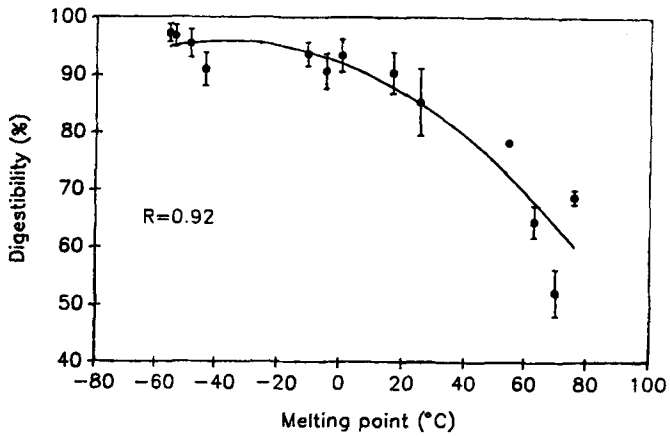

FIG. 3. Digestibility (%) in Atlantic salmon of fish oil fatty acids fed as triacylglycerols, plotted as a function of their melting point. Data are the mean ± SD of four determinations. For one point, the SD was too small to mark.

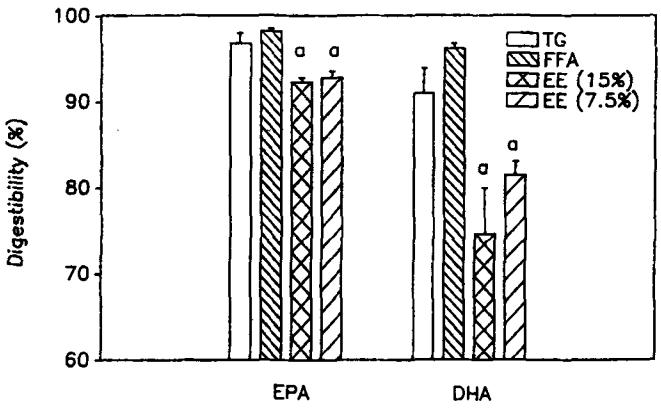

FIG. 4. Digestibility in Atlantic salmon of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in different chemical forms; triacylglycerols (TG), free fatty acids (FFA), and ethyl esters (EE). Data are the mean ± SD of four determinations, except for the group fed 15% ethyl esters (two determinations). [a]Mean is significantly ($P < 0.05$) different from mean digestibility when feeding free fatty acids and triacylglycerols.

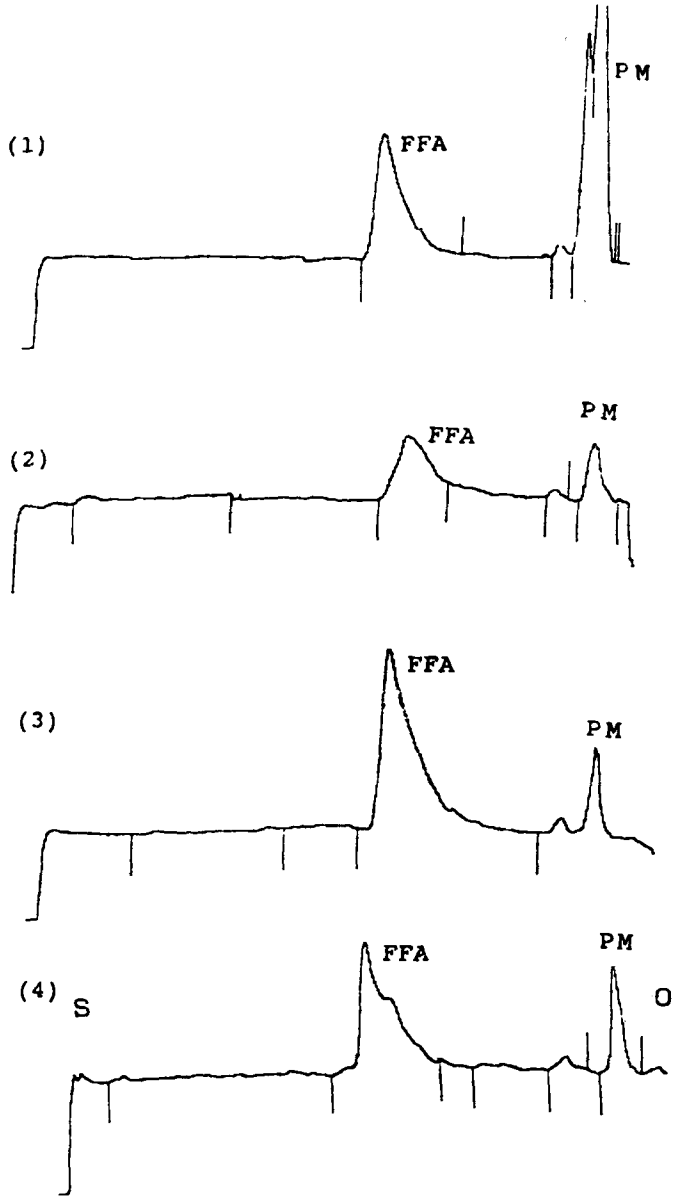

FIG. 5. TLC/FID chromatograms of the classes in lipids of feces of Atlantic salmon when fed; (1) triacylglycerols (TG), (2) free fatty acids (FFA), (3) ethyl esters (EE) and (4) a 1:1 mixture of triacylglycerols and ethyl esters (TG/EE). PM refers to polar materials, all soluble in chloroform and, hence, lipids. The origin of scan (0) and stop at end (S) are marked. Ethyl esters (not shown) are more mobile than FFA on silica gel Chromarods and were only observed in the one feces sample from fish with diarrhea. Triacylglycerols (not shown) were not observed, but would have appeared between PM and FAA.

TABLE 5

Dry Weight and Lipid Content of Feces from Atlantic Salmon, Fed Four Different Diets[a]

Diet	Dry weight (%)[b]	Lipid (%dwb)[c]
1	13.5 ± 0.8	6.1 ± 0.7
2	10.9 ± 1.3	5.2 ± 1.0
3	16.8 ± 1.5	6.2 ± 0.1
4	15.9 ± 2.5	9.1 ± 0.8

[a]Diet 1, triacylglycerols; Diet 2, free fatty acids; Diet 3, 15% ethyl esters; and Diet 4, 7.5% ethyl esters/7.5% corn oil.
[b]Data are the mean ± SD of 4 determinations.
[c]Data are the mean ± SD of 2-4 determinations.

esters, are not absorbed as well by Atlantic salmon as the same fatty acids fed in free acid form or as triacylglycerols. Subsequent deposition of fatty acids from ethyl esters, for example, in muscle phosphatidylcholine (41), appeared to be normal (12). The preferred form for concentrate administration should therefore be free fatty acids. Cholestane is especially convenient and useful as a marker for studying the digestibility of fatty acids in any form, and lends itself especially well to total saponification of feces *in lieu* of lipid extraction (42).

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Comparison of the Effects of Dietary Fish Oils with Different n-3 Polyunsaturated Fatty Acid Compositions on Plasma and Liver Lipids in Rats

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The effects of dietary fish oils with different n-3 polyunsaturated fatty acid compositions on plasma lipid profiles in rats have been studied. Forty-eight male rats, previously maintained on a cholesterol-free diet for 15 days, were fed for 60 days with diets supplemented with 10% fat of either marine hilsa fish (*Hilsa ilisa*, family clupeidae) or fresh-water chital fish (*Notopterus chitala*, family notopteridae). The diets had similar levels of total saturated (35–41%), monounsaturated (43–47%) and n-3 polyunsaturated (9–10%) fatty acids. Cholesterol contents of the diets were adjusted to 0.85%; γ-linolenic acid (3.3%) in chital oil and eicosapentaenoic acid (4.9%) in hilsa oil diets were the major n-3 contributors. The percentage of eicosapentaenoic acid in the chital oil diet was 0.57 times that of the hilsa oil diet, but the eicosapentaenoic (EPA) to arachidonic acid (AA) ratio in the latter (4.08) was 3.2 times that of the former (1.27). Sixty days of hilsa oil diet feeding decreased the levels of cholesterol (53.3 ± 2.9 to 50.0 ± 1.1 mg/dL), triacylglycerol (75.7 ± 3.8 to 64.3 ± 2.6 mg/dL) and phospholipid (55.8 ± 1.5 to 51.7 ± 3.1 mg/dL) in rat plasma. Similar treatment with chital oil diet elevated the plasma cholesterol level (53.3 ± 2.9 to 62.3 ± 7.6 mg/dL) while triacylglycerol and phospholipid contents remained unaltered. Both the dietary treatments decreased the levels of linoleic and arachidonic acids in liver but only under the hilsa oil diet did the eicosapentaenoic acid percentage increase markedly (0.8 ± 0.06% to 5.5 ± 0.06%) at the expense of arachidonic acid. This study strongly suggests that the hypolipidemic effect depends on the composition of the n-3 polyunsaturated fatty acids rather than on the total n-3 polyunsaturated fatty acid content of the dietary fish oil.
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A high plasma cholesterol level and the occurrence of coronary heart disease are strongly interrelated (1). Dietary supplementation with polyunsaturated fat is associated with a reduction in the incidence of occlusive vascular diseases including both atherosclerosis and thrombosis (2,3). Administration of marine fish oils rich in n-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), decreased plasma cholesterol and triacylglycerol contents in normolipidemic and hyperlipidemic human subjects (4–6) and also in rats (7–12). Feeding of such oils was found to reduce platelet aggregability (4,5,13–15), thus lowering the chance of thrombosis, a major factor in heart attacks and strokes.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; NEFA, non-esterified fatty acids; PUFA, polyunsaturated fatty acids; RT, retention time.

The n-3 PUFA of dietary fish oils are generally considered the main causative factor for the aforesaid hypolipidemic effect, but the data so far obtained are still insufficient to determine whether this effect is due to an increased intake of n-3 PUFA or due to the specific distribution of the three prostaglandin-producing PUFA, dihomo-γ-linolenic acid (20:3n-6), arachidonic acid (AA; 20:4n-6), and EPA (20:5n-3) of fish oil.

In this communication, we compare the plasma lipid lowering effects of the oils of two fishes, one of marine and the other of fresh water origin. Both fish oil diets contained similar amounts of total saturated acids (principally 16:0), total monunsaturated acids (mainly 16:1 and 18:1), n-3 PUFA, docosapentaenoic acid (DPA; 22:5n-3) and DHA (22:6n-3). So it is expected that the effects exerted by these factors on plasma lipid profile would be the same for the two fish oil supplemented diets. But the EPA/AA ratios of these two oils differ considerably. Our aim was to find out whether the effectiveness of a dietary fish oil in producing the hypolipidemic effect depends upon the quantity or the type of n-3 PUFA in the oil.

MATERIALS AND METHODS

Fish and fish oil. Hilsa fish (*Hilsa ilisa,* English hilsa shad) of the clupeidae family were collected from the river Ganges near Calcutta. Chital fish *(Notopterus chitala)* of the notopteridae family were purchased from a Calcutta market. Both fish were collected during the months of June to August, and are considered excellent food sources.

Oils were extracted from the flesh of both fish. Flesh only from the belly flap of chital fish was used because this portion of the body is richer in fat content than the rest. By contrast, fat in hilsa fish is more or less evenly distributed. Hilsa fish with minimum egg content were selected because egg formation depletes the lipid reserves of the fish (16).

Chopped fish flesh was extracted with acetone (1 L/kg) in a Soxhlet extractor for 8–10 h, the resulting emulsion was broken by adding 250 mL each of chloroform and water. The lower phase containing the fat was washed three times with water and dried overnight over anhydrous Na_2SO_4. Solvent was removed under reduced pressure and finally by flushing with N_2. The oil was stored in air-tight amber-colored bottles under N_2 at −20°C. A nitrogen atmosphere was maintained, whenever possible, during the entire operation to minimize oxidation of the oil.

Total fat contents of wet flesh of chital and hilsa fish were 8.38% and 15.9%, respectively. Cholesterol contents of the oils were 8.5% and 8.1% for chital and hilsa oils, respectively. Cholesterol (0.4 g) was added to each 100 g of hilsa oil in order to make the two dietary oils equal in cholesterol content.

Animals and diets. Fifty-four male Charles Foster rats (100 g, average weight) were maintained on a semisynthetic standard diet for 15 days. The composition of the diet in weight percent was casein 20, starch 40, sucrose

20.15, cellulose 5, U.S.P. salt mix XIV 4.5, vitamin mix 0.15 (17), choline chloride 0.2, BHT 0.002, and groundnut oil 10. Starch, cellulose and casein were defatted by Soxhlet extraction with *n*-hexane for 12 h. This dietary treatment was meant to standardize the animals before starting the actual experiments with fish oil supplemented diets. After 15 days, 6 rats were killed for analyses (these are the 0-day data in the tables). The remaining 48 rats were divided into two groups of 24 rats each, such that the average weights per group were similar. Experimental diets were prepared by adding 10 g of either hilsa or chital oil to 90 g of fat-free basal diet mix as described above. Fatty acid compositions of the three diets are presented in Table 1.

Animals were housed individually in cages in an airconditioned animal house (20–23°C) and fed experimental diets *ad libitum* for 60 days. Food was given twice a day (8 a.m. and 8 p.m.); uneaten food was discarded. Diets were prepared twice a week and stored under a N_2 atmosphere at −20°C. Food intake and body weights were recorded every other day. On the 15th, 30th, 45th and 60th days of feeding, 6 rats from each dietary group were killed for analyses.

Lipid analysis. The animals were fasted for 12 h and killed by drawing blood from the abdominal aorta under light diethyl ether anaesthesia. Blood was collected into plastic syringes and anticoagulated with 3.8% trisodium citrate (9 vol blood and 1 vol citrate). Plasma was separated by centrifuging the blood at 1500 × *g* for 10 min. Plasma from 2 rats was pooled together so that 3 sets of data were produced for each group. Whenever necessary, liver tissue was excised, rinsed thoroughly with ice-cold physiological saline and kept frozen at −20°C until analysis. An aliquot of plasma was extracted using a one-step procedure (18) for quantitation of plasma lipids. Total cholesterol, phospholipid and triacylglycerol contents of plasma were determined using standard procedures (19–21). Plasma non-esterified fatty acid (NEFA) concentration was measured using a micromethod (22), developed in this laboratory.

For fatty acid analyses, plasma and liver tissues were extracted according to the method of Bligh and Dyer (23). Total lipids were transmethylated in the presence of 5% methanolic HCl (24).

Fatty acid compositions were determined by gas-liquid chromatography of methyl ester using 15% diethyleneglycol-succinate polyester on 100–120 mesh Gaschrom W (Hewlett-Packard, Avondale, PA) columns, packed in 6 ft × 1/8 inch (o.d.) stainless-steel tubes. The flow rate of the carrier gas (nitrogen) was 30 mL/min; the injection port and detector were maintained at 200°C. The column temperature was held at 170°C for 1 min and then programmed at 2.5°/min to 200°C. A dual-column, fully computerized gas chromatograph with dual flame-ionization detectors (FID) (Hewlett-Packard, Model 5840A) was used. The compositions in Tables 1, 3 and 4 are expressed as weight percentages (wt%) and represent the average of three separate analyses. Fatty acid peaks were identified by comparing the corresponding retention times (RT) with those of standard methyl esters.

Statistical analysis of data. The results are expressed as mean ± SD. Statistical significance was analyzed by Student's *t*-test.

TABLE 1

Fatty Acid Composition (wt%) of the Experimental Diets[a]

Fatty acids	Groundnut oil	Chital oil	Hilsa oil
16:0	17.2	29.4	33.0
16:1	trace	12.0	17.7
17:0 + 18:0 iso	trace	trace	1.5
18:0	2.7	5.6	6.7
18:1	44.5	30.9	29.6
18:2n-6	32.8	3.4	0.3
18:3n-6	trace	0.1	0.4
18:3n-3	2.7	3.3	0.7
18:4n-6	trace	3.1	0.7
18:4n-3	trace	trace	0.4
20:2n-6	trace	0.4	trace
20:3n-6	trace	0.7	0.2
20:4n-6	trace	2.2	1.2
20:4n-3	trace	0.9	0.5
20:5n-3	trace	2.8	4.9
22:4n-6	trace	1.0	0.4
22:5n-6	trace	1.2	0.3
22:5n-3	trace	1.4	1.0
22:6n-3	trace	1.6	1.5
Total			
Saturated	19.9	35.0	41.2
Monounsaturated	44.6	42.9	47.3
n-3 PUFA	2.7	10.0	9.0
Ratio			
n-3/n-6	0.08	0.83	2.81
EPA/AA	—	1.27	4.08

[a]The fat-free basal diet was supplemented with 10% by weight of the respective oils. The basal diet contained the following components (g/kg diet): casein, 200; starch, 400; sucrose, 201.5; cellulose, 50; U.S.P. salt mix. XIV, 45; vitamin mix, 1.5 (17), choline chloride, 2.0; BHT, 0.02. At least three random samples of each diet were analyzed; trace, trace amount (less than 0.1%).

RESULTS

The fatty acid compositions of the diets are presented in Table 1. Groundnut oil supplemented standard diet contained mainly oleic acid (18:1, 44.5%) and linoleic acid (18:2n-6, 32.8%). The n-3 fatty acid contents of both fish oil diets were essentially the same (approximately 10%). However, the chital oil diet was high in γ-linolenic acid (18:3n-3), while the hilsa oil diet was rich in EPA.

The fish oil diets differed considerably in n-3/n-6 ratios (0.83 in chital oil diet and 2.81 in hilsa oil diet) and EPA/AA ratios (1.27 in chital oil diet and 4.08 in hilsa oil diet).

The fatty acid composition of the hilsa oil diet closely resembles that of the Eskimo diet (25,16), being rich in monoenoic acid (57.3%), but low in polyenoic (19.2%) fatty acids, has a very low AA content (0.4%), and contains almost the same proportion of EPA as does the hilsa oil diet (4.6% in Eskimo diet and 4.9% in hilsa oil diet).

Body weights and food consumption. All rats appeared healthy after the 8-week feeding regimen. There was no significant difference in weight gain under the two different fish oil treatments, and food consumption during the feeding trial was also similar (data not shown).

Lipid contents of rat plasma. Plasma total cholesterol, triacylglycerol and total phospholipid contents significantly increased within 15 days in rats fed the fish oil diets (Table 2). However, on the 60th day total cholesterol,

TABLE 2

Effect of the Two Fish Oil Diets on Plasma Total Cholesterol, Triacylglycerol, Total Phospholipid and Non-Esterified Fatty Acid Concentration of Rats[a]

Lipids	0-day	Chital oil Days on test				Hilsa oil Days on test			
		15	30	45	60	15	30	45	60
Total cholesterol (mg/dL)	53.3[b] ± 2.9	90.0[c] ± 17.3	66.7 ± 5.8	61.7 ± 2.9	62.3 ± 7.6	86.7[c] ± 11.5	55.0[b] ± 7.1	50.3[b] ± 11.5	50.0 ±1.1
Triacylglycerol (mg/dL)	75.7[b] ± 3.8	83.7[c] ± 5.3	77.7[b,c] ± 3.3	73.9[b,c] ± 7.5	75.3[b] ± 1.5	84.6[c] ± 6.3	75.0[b,c] ± 4.8	67.0[b,c] ± 3.5	64.3 ± 2.6
Total phospholipid (mg/dL)	55.8[b] ± 1.5	66.1 ± 3.9	55.0[b,c] ± 1.8	55.8[b] ± 2.5	56.3[b] ± 1.9	60.8 ± 3.8	53.6[b,c] ± 3.0	51.8 ± 3.2	51.7 ± 3.1
Nonesterified fatty acids (μmole/L)	0.82[b] ± 0.20	1.08[b,c] ± 0.30	1.04[b] ± 0.30	1.025 ± 0.10	1.03 ± 0.20	0.87[b,c] ± 0.20	0.75[b] ± 0.04	0.67[b] ± 0.10	0.68[b] ± 0.03

[a]Each value represents the mean ± SD; n = 6; $P < 0.05$.

[b]Non-significance of the difference between 0-day data and that due to each fish oil feeding.

[c]Non-significance of the difference between the two fish oil feeding data for a specific day.

triacylglycerol, total phospholipid and NEFA became significantly lower under the hilsa oil dietary regimen than they were upon chital oil feeding. The lowering of cholesterol was significant even on the 30th day of the experiment.

Fatty acid composition of plasma and liver lipids. The 18:2n-6 and 20:4n-6 contents of plasma total lipids under the two fish oil diets were very similar (Table 3), even though chital oil contains about 10 times more 18:2n-6 (3.4%) than does hilsa oil (0.3%). Therefore, a certain level of essential fatty acids, 18:2n-6 and AA, was retained in the plasma within the experimental period, despite very low percentages of these two fatty acids in the hilsa oil diet.

Though both dietary fish oils significantly reduced AA content of liver total lipids (Table 4), the extent of the decrease was larger with the hilsa oil diet, which has a higher EPA/AA ratio in comparison to the chital oil diet.

TABLE 3

Fatty Acid Compositions of Rat Plasma Total Lipids After 60 Days of Feeding Fish Oil Diets[a]

Fatty acids	0-day	Chital oil	Hilsa oil
16:0	20.9 ± 0.9	28.8 ± 0.8[b]	29.4 ± 2.0[b]
16:1	3.0 ± 0.5	4.6 ± 0.9	6.2 ± 0.5
17:0	trace	0.5 ± 0.06	trace
18:0 iso	0.3 ± 0.1	0.5 ± 0.1[b]	0.7 ± 0.2[b]
18:0	12.4 ± 1.1[b]	11.8 ± 0.06[b]	9.3 ± 1.3
18:1	20.9 ± 2.3	24.8 ± 2.1[b]	23.5 ± 0.15[b]
18:2n-6	16.7 ± 0.9	7.3 ± 1.1[b]	6.1 ± 0.4[b]
18:3n-3	0.2 ± 0.1	1.0 ± 0.10	0.4 ± 0.05
18:4n-6	0.7 ± 0.06[b]	trace	0.8 ± 0.2[a]
18:4n-3	trace	trace	trace
20:2n-6	trace	trace	0.1 ± 0.0
20:3n-6	0.3 ± 0.1[b]	0.8 ± 0.1	0.2 ± 0.06[b]
20:4n-6	21.2 ± 1.0	13.3 ± 0.7[b]	13.4 ± 2.1[b]
20:4n-3	trace	trace	trace
20:5n-3	trace	2.0 ± 0.2	4.8 ± 1.5
22:4n-6	1.0 ± 0.5[b]	1.0 ± 0.3[b]	1.1 ± 0.15[b]
22:5n-6	0.9 ± 0.1	0.5 ± 0.1[b]	0.6 ± 0.1[b]
22:5n-3	0.2 ± 0.0	1.0 ± 0.5[b]	1.0 ± 0.2[b]
22:6n-3	1.3 ± 0.1	2.0 ± 0.6[b]	2.4 ± 0.06[b]
Total			
n-6 PUFA	40.8	22.9	22.3
n-3 PUFA	1.7	6.0	8.6
Ratio			
n-3/n-6	0.04	0.26	0.38
EPA/AA	—	0.15	0.36

[a]Each value represents mean ± standard deviation of three separate determinations. Values without a common superscript are significantly different ($P < 0.05$); trace, trace amount.

TABLE 4

Fatty Acid Compositions of Rat Liver Total Lipids After 60 Days of Feeding Fish Oil Diets[a]

Fatty acids	0-day	Chital oil	Hilsa oil
16:0	23.1 ± 2.2[b]	25.5 ± 0.9[b]	25.0 ± 1.3[b]
16:1	1.3 ± 0.9	7.8 ± 1.0	5.7 ± 0.2
17:0	trace	1.0 ± 0.3	0.6 ± 0.1
18:0 iso	trace	1.1 ± 0.1	0.9 ± 0.1
18:0	25.8 ± 1.0	15.6 ± 3.2[b]	19.9 ± 2.1[b]
18:1	12.4 ± 0.7	18.0 ± 2.5	23.6 ± 1.3
18:2n-6	15.3 ± 1.3	4.9 ± 0.2	3.0 ± 0.9
18:3n-3	0.3 ± 0.1[b]	0.9 ± 0.1	0.5 ± 0.2[b]
18:4n-6	trace	trace	0.2 ± 0.0
18:4n-3	trace	trace	trace
20:2n-6	0.5 ± 0.2	0.1 ± 0.0	0.2 ± 0.1
20:3n-6	0.3 ± 0.1	1.0 ± 0.06	0.9 ± 0.1
20:4n-6	16.1 ± 1.1	13.1 ± 1.1	8.6 ± 1.1
20:4n-3	trace	trace	0.1 ± 0.0
20:5n-3	0.8 ± 0.06	1.2 ± 0.1	5.5 ± 0.06
22:4n-6	1.7 ± 1.0[b,c]	2.4 ± 0.2[b]	1.1 ± 0.3[c]
22:5n-6	1.0 ± 0.1	2.5 ± 0.1	0.6 ± 0.1
22:5n-3	0.3 ± 0.1	2.0 ± 0.2	1.4 ± 0.2
22:6n-3	1.1 ± 0.9[c]	2.9 ± 0.4[b]	2.2 ± 0.5[b,c]
Total			
n-6 PUFA	34.9	24.0	14.6
n-3 PUFA	2.5	7.0	9.7
Ratio			
n-3/n-6	0.07	0.29	0.66
EPA/AA	0.05	0.09	0.64

[a]Each value represents mean ± standard deviation of three separate determinations. Values without a common superscript are significantly different ($P < 0.05$); trace, trace amount.

EPA is the major n-3 PUFA in hilsa oil but not in chital oil. There was a considerable difference in the EPA content of liver lipids under the two fish oil diets (5.5 ± 0.6% for hilsa oil diet and only 1.2 ± 0.1% for chital oil diet). As a result, the EPA/AA ratio in the liver total lipids under hilsa oil treatment was about 7 times higher than it was under chital oil treatment.

DISCUSSION

Several studies have demonstrated the effects of n-3 PUFA, especially EPA and DHA, on plasma lipid profile, platelet function and eicosanoid formation (6,14,25-27). However, in fish oil feeding studies different fish oils containing various types and amounts of n-3 PUFA have been used, making a true evaluation of the effect of any particular n-3 PUFA difficult. The present experiments were carried out to study the effect of a specific composition of n-3 PUFA on the plasma and liver lipid profiles in rats. The hypolipidemic effects of two fish oil diets having similar proportions of total saturated acids, monounsaturated acids, n-3 PUFA, 22:5n-3, and 22:6n-3, and the same amounts of cholesterol (adjusted), were compared. The fish oils were chosen so they would differ considerably in their EPA/AA ratios.

We found a substantial difference between the effects of these two fish oil diets on plasma lipid profiles. Hilsa oil diet decreased the total cholesterol, triacylglycerol, phospholipid and NEFA contents to significantly lower levels than did the chital oil diet. This difference is most noticeable between the plasma total cholesterol levels, although the same amount of cholesterol was present in the two diets.

The hilsa oil diet increased the EPA level and decreased the AA level in liver lipids. These changes were more pronounced than the corresponding changes due to the chital oil diet, although both diets contained similar amounts of n-3 PUFA. The main difference between the fatty acid composition of these two fish oils is in their EPA/AA ratios. Although the level of EPA in hilsa oil is not very high (4.9%), the EPA/AA ratio in hilsa oil (4.08) is 3.2 times higher than that of chital oil (1.27). Thus, the higher EPA/AA ratio in the hilsa oil diet appears to have facilitated the greater enrichment of tissue lipids with EPA at the expense of AA than was observed with the chital oil diet. The study also pointed out the importance of n-3 PUFA composition over PUFA amount to assess the hypolipidemic effect of a fish oil diet.

Diets with a high PUFA content have been recommended by several medical and nutritional boards because of the lipid-lowering effect of PUFA in plasma. However, the high PUFA containing fish oils are apt to be oxidized during extraction, purification and storage. Cost-effective measures, such as processing under a nitrogen atmosphere and the addition of antioxidants (vitamin E, BHT), can minimize oxidation. Hilsa fish oil is lower in total PUFA content than are other fish oils, but it is a good source of C_{20} and C_{22} n-3 PUFA. The moderate n-3 PUFA content of this oil is sufficient to exert its hypolipidemic effects. The low total PUFA content of this oil would also be expected to make it less susceptible to autoxidation and should therefore better lend itself to food processing than would other highly unsaturated fish oils.

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Abnormal Lipid and Fatty Acid Compositions of Kidneys from Mice with Polycystic Kidney Disease

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Renal cyst development in polycystic kidney disease (PKD) involves hyperplastic growth and extensive membrane alterations, suggesting abnormal membrane composition and function. Using thin-layer and gas-liquid chromatography, we analyzed the lipid components of the kidneys from 120-day-old DBA/2FG-*pcy (pcy)* having PKD as compared to normal DBA/2J (DBA) mice. At sacrifice, kidneys from *pcy* mice were four times larger than DBA controls, indicating that extensive renal cyst growth had occurred. The ratios of cholesterol/phospholipid, choline glycerophospholipid (GPC)/ethanolamine glycerophospholipid (GPE) and alkenylacyl GPE/diacyl GPE were higher (by 25%, 41% and 72%, respectively) in the cystic kidneys, while total phosphatidylinositol (PI), GPE and cardiolipin (DPG) were lower (by 13%, 23% and 27%, respectively). With respect to fatty acid compositions, there were significantly lower levels of docosahexaenoic acid (DHA, 22:6n-3) and higher levels of adrenic acid (AdA, 22:4n-6) in the phospholipids of *pcy* mouse kidneys. These changes were not present in serum, indicating that they were not generalized differences. Interestingly, the lower level of DHA in GPE was found to be associated with the alkenylacyl, but not the diacyl species. The fatty acids comprising the product/substrate ratio for the Δ4 desaturase activity were lower across all phospholipids, indicating a possible abnormality in polyunsaturated fatty acid metabolism in this model of PKD. These lipid abnormalities may influence membrane-mediated events such as receptor activation, signal transduction, ion transport and enzyme activities. The renal pathophysiologies associated with PKD may be related to the tissue lipid abnormalities described herein.

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Cell proliferation is influenced by membrane properties such as enzyme activities, permeability, ion transport and fluidity which are influenced by the phospholipid environment (1–3). As a result, altered lipid composition is seen in abnormal cellular proliferation such as in tumor growth. In particular, factors known to influence membrane fluidity, such as docosahexaenoic acid (DHA, 22:6n-3) levels in membrane phospholipid and the cholesterol/phospholipid ratios, have been shown to be altered in tumor cells (4–8). Phospholipid methylation also appears to be increased in tumor cells (9), and elevated choline glycerophospholipid (GPC)/ethanolamine glycerophospholipid (GPE) ratios have been demonstrated in high metastatic compared to low metastatic cell lines (10,11). In addition, tumor cells often exhibit altered metabolism of ether lipids and increased content of this unique class of lipid (8, 12–16).

In polycystic kidney disease (PKD), renal tissue becomes grossly enlarged as cysts proliferate in all parts of the nephron, particularly the tubules (17). Although these cysts are filled with fluid instead of cellular material, renal cysts are comparable to slow-growing adenomas in many respects (18). In addition to fluid accumulation, epithelial cell hyperplasia is an essential feature of PKD (19,20). Hyperplasia of epithelial cells has been shown in many cysts (19,21,22), and it has been reported that the earliest changes noted in the development of cysts are proximal tubule cell hyperplasia and tubule dilatation (23,24). Polyps are also a common feature of cyst epithelial cells in both genetic and non-genetic forms of the disease, as well as chemically-induced models of PKD (19,21,25–28). Cystic lesions have also been seen in kidneys of rats fed a carcinogen to induce renal adenocarcinoma (29).

The recent availability of a mouse model of PKD (30,31) provides an opportunity to evaluate any lipid abnormalities in the kidneys of these diseased animals. Since cyst development involves hyperplastic growth and extensive membrane alterations, we have determined the lipid characteristics of the kidneys from DBA/2FG-*pcy (pcy)* mice (including individual phospholipids, their subclasses and fatty acid profiles) as compared to the corresponding DBA/2J (DBA) controls. The present findings indicate several lipid abnormalities in this model of PKD, including alterations in cholesterol/phospholipid and GPC/GPE ratios, DHA levels in individual phospholipids and ether phospholipid compositions.

EXPERIMENTAL PROCEDURES

Animals and lipid extraction. Male DBA and *pcy* mice (n = 4 per group) were weaned at 30 days and maintained on lab chow (Ralston Purina, St. Louis, MO) until 120 days of age, at which time the animals were sacrificed following an overnight fast. Blood samples were taken by heart puncture and the serum was frozen in liquid nitrogen. The kidneys were also removed, weighed and frozen. The right kidneys were homogenized in 5 mL of chloroform/methanol (2:1, vol/vol) and lipids from both kidney and serum were extracted by the Folch method (32). Known aliquots of the lipid extracts were taken for analysis of total phospholipid and triacylglycerol (TG), individual phospholipids and the subclasses of GPC and GPE.

Lipid analysis. Following saponification of the lipid extracts, serum and renal cholesterol concentrations were determined enzymatically using the Cholesterol High Performance CHOD-PAP method (Boehringer Mannheim Canada, Dorval, Quebec, Canada).

For analysis of renal and serum total phospholipid and TG, a measured aliquot of the lipid extract was spotted on Merck silica gel 60HR thin-layer chromatography

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Abbreviations: AdA, adrenic acid; DBA, DBA/2J normal mice; DHA, docosahexaenoic acid; DPG, diphosphatidylglycerol (cardiolipin); FAME, fatty acid methyl esters; GLC, gas-liquid chromatography; GPC, choline glycerophospholipid; GPE, ethanolamine glycerophospholipid; *pcy*, DBA/2FG-*pcy* diseased mice; PI, phosphatidylinositol; PKD, polycystic kidney disease; PS, phosphatidylserine; SPH, sphingomyelin; TG, triacylglycerol; TLC, thin-layer chromatography.

(TLC) plates (British Drug House, Toronto, Ontario, Canada), and developed in heptane/iso-propyl ether/acetic acid (60:40:3, by vol) (33). Chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol) (34) was used as the developing solvent to separate the individual phospholipids in another measured aliquot of the lipid extract. Fatty acid methyl esters (FAME) were then prepared and analyzed by gas-liquid chromatography (GLC) as described (34). Phospholipids were quantified by dividing the amount (in nmol) of fatty acids for each fraction by the number of fatty acids per molecule, taking into consideration that the alkylacyl or alkenylacyl phospholipid subclasses contain only one fatty acid per molecule whereas the diacyl contain two.

GPC and GPE subclass analysis. The subclasses of GPC and GPE were analyzed by a modification of the method of Renkonen (35). Briefly, GPC and GPE were separated by TLC and eluted as described (36). Each extract was evaporated under nitrogen and the residual phospholipid was resuspended in 1 mL of diethyl ether. To this solution was added 1.5 mL of a 17.5 mM Tris buffer (pH 7.3) containing 40 units of phospholipase C (*Bacillus cereus,* type V; Sigma Chemical Co., St. Louis, MO) and 0.5 mL 1% $CaCl_2$ (37). This mixture was incubated by gentle agitation at 37°C for 16 h to completely hydrolyze all three subclasses of phospholipid. The resulting diradylglycerols produced were extracted 3 times with 2 mL diethyl ether; following solvent evaporation, they were resuspended in 1 mL acetic anhydride and 4–5 drops of pyridine to produce diradylglycerol acetates. After heating for 75 min at 80°C, 1 mL of methanol was added (on ice). This mixture was completely dried down, and 2 mL of water and 3 mL of diethyl ether/methanol/acetic acid (60:40:1, by vol) were added. The diradylglycerol acetates were extracted twice with 3 mL of petroleum ether (b.p. 38–56°C) and subsequently separated by triple-development TLC. The first and third developments were in toluene and the second development was in hexane/diethyl ether (4:1, vol/vol). This system readily separates the 1,2-diacyl, any 1,3-diacyl, alkylacyl and alkenylacyl glycerol acetates produced (R_f values of 0.25, 0.33, 0.39 and 0.47, respectively). Only trace amounts of 1,3-diacyl glycerol acetate were detected, indicating that isomerization of the diradylglycerol and diradylglycerol acetates was minimal during this procedure. The resolved bands were scraped into test tubes containing monopentadecanoic acid as an internal standard, and the fatty acids were analyzed as FAME following transmethylation (34). For the alkenylacyl fractions, TLC was used to separate the FAME from the dimethyl acetals derived from the vinyl ether linked fatty aldehydes. After developing the TLC plate in toluene, the FAME were eluted from the gel scrapings by adding 2 mL methanol, 2 mL petroleum ether and 1 mL water, and after removing the upper phase, were dissolved in hexane for GLC.

Statistical analysis. Statistical differences between data obtained from *pcy* (diseased) and DBA (control) mouse kidneys and serum were determined using Student's unpaired *t*-test, except the GPC/GPE ratios and kidney weight data, which were compared using the Mann-Whitney test for non-parametric data. Significance of correlations was determined using Fisher's tables (38).

RESULTS AND DISCUSSION

At sacrifice, kidneys from *pcy* mice were obviously enlarged and discolored compared to normal DBA kidneys. Overall, the *pcy* (right) kidneys were four times larger than the DBA controls (means ± SE: 0.51 ± 0.13 g for *pcy* compared to 0.12 ± 0.01 g for DBA, $P < 0.05$), indicating that extensive renal cyst growth had already occurred in these mice.

Total phospholipid, TG and cholesterol levels were similar (as nmol/kidney) in *pcy* compared to DBA kidneys (Table 1). On the other hand, total phospholipid and cholesterol levels were higher (by 123% and 35%, respectively) in serum from *pcy* mice, while TG levels were similar (Table 1). Although it has not been specifically shown in human PKD (39), elevated serum lipids can contribute to impaired renal functioning (40,41), and the elevated serum lipid levels seen herein may possibly be associated with the development of renal deterioration in *pcy* mice.

The higher cholesterol/phospholipid ratio in the *pcy* kidney (by 25%, Table 1) is similar to that reported for tumor cells (5–7). Membrane viscosity is increased with increased cholesterol/phospholipid levels (6,42); thus membrane-mediated events related to cell growth, such as receptor activation, signal transduction and ion transport, possibly may be altered as a result.

Table 2 gives the phospholipid compositions of the DBA and *pcy* mouse kidneys. Lower levels of GPE, phosphatidylinositol (PI), cardiolipin (diphosphatidylglycerol, DPG) and a higher GPC/GPE ratio were found in *pcy* mouse kidneys compared to normal kidneys. The lower levels of PI seen in *pcy* kidneys possibly may be of relevance to the recognized involvement of inositol phospholipid metabolism in membrane signalling associated with cell proliferation and growth (43), as well as the

TABLE 1

Renal and Serum Lipid Composition of DBA and *pcy* Mice[a]

	nmol/Kidney		nmol/100 mL Serum	
	DBA	*pcy*	DBA	*pcy*
Total phospholipid	5054 ± 138	4766 ± 358	116.5 ± 8.3	259.3 ± 4.4[b]
Total cholesterol	1597 ± 45	1920 ± 177	177.0 ± 9.0	239.0 ± 2.4[b]
TG	1582 ± 171	1466 ± 737	74.9 ± 14.9	61.3 ± 11.0
Cholesterol/phospholipid	0.32 ± 0.00	0.40 ± 0.01[b]	1.54 ± 0.11	1.50 ± 0.06

[a]Values represent means ± SE (n = 4).

[b]Significantly different frm DBA, $P < 0.001$.

TABLE 2

Renal Phospholipid Composition of DBA and *pcy* Mice[a]

	nmol/Kidney	
	DBA	*pcy*
GPC	2029 ± 87	2188 ± 237
GPE	1362 ± 64	1054 ± 70[b]
PI	343 ± 9	297 ± 9[b]
PS	393 ± 31	335 ± 35
DPG	255 ± 4	187 ± 18[c]
SPH	671 ± 29	706 ± 97
GPC/GPE	1.50 ± 0.07	2.11 ± 0.30[b]

[a]Values represent means ± SE (n = 4).
[b]Significantly different from DBA, $P < 0.05$.
[c]Significantly different from DBA, $P < 0.01$.

generation of two intracellular second messengers, diacylglycerol [a protein kinase C activator (44)] and inositol trisphosphate [involved in intracellular Ca^{++} mobilization (45)]. The lower levels of DPG (by 27%) may reflect cellular/mitochondrial differences (see below). The higher GPC/GPE ratio found in *pcy* kidneys is also of interest because elevated GPC/GPE ratios have been reported in high-metastatic tumor cells (10,11).

The relative amounts of diacyl GPE and alkenylacyl GPE were also altered in *pcy* kidneys (Table 3): Calculations from Tables 2 and 3 show that diacyl GPE is 36% lower in *pcy* kidneys, while the level of alkenylacyl GPE is conserved, resulting in a relative enrichment of this ether phospholipid. The relatively higher level of ether lipid content in *pcy* kidneys follows a general trend of ether lipid enrichment in neoplastic cells (8,12–16). While the biochemical function of plasmalogenic phospholipid (specifically alkenylacyl GPE) has not been fully elucidated, it has been suggested to function as a cellular antioxidant (46). Lipid peroxidation and free radical formation recently have been implicated in the progression of renal disease (47,48). Alkenylacyl GPE may therefore have a function in PKD in relation to oxidative tissue damage, or may be related to alterations in membrane packing.

With respect to the GPC/GPE ratio and the two major subclasses of GPE, namely diacyl GPE and alkenylacyl GPE, significant correlations ($P < 0.05$) with kidney weight were noted in *pcy* kidneys. The relative proportion of GPC (total), as well as the GPC/GPE ratio were found to correlate positively with kidney weight (cystic involvement). Conversely, the relative proportion of GPE (total) correlated negatively with kidney weight (data not shown). With respect to the GPE subclasses, the proportion of alkenylacyl GPE and diacyl GPE correlated positively and negatively, respectively, with kidney weight. The relative levels of these classes of phospholipids may therefore be used as possible markers for the extent of cystic involvement in *pcy* kidneys.

The fatty acid profile of the DBA kidney total phospholipid (Table 4) is in general agreement with previous reports on mouse kidney (49,50). While minor differences were found in various fatty acids, of particular interest was the finding of significantly lower levels of DHA in the *pcy* mouse kidney (by 22% overall) and higher levels of adrenic acid (AdA, 22:4n-6). Minor differences also were observed in the TG fraction. The corresponding data for the serum phospholipid and TG (Table 5) did not mimic the differences seen in mouse kidney, indicating that the renal abnormalities are not generalized. No significant difference was found in the level of DHA in the serum phospholipid, and a moderately higher level of DHA in serum TG was found in *pcy* relative to DBA animals. The lower amounts of DHA in the renal phospholipid from the *pcy* animals may reflect an impaired uptake and entry of DHA into kidney phospholipid, and/or an impaired conversion of α-linolenic acid to DHA *via* desaturation/elongation reactions and/or altered retroconversion of DHA.

The most consistent alterations in fatty acid compositions in *pcy* mouse kidneys across individual phospholipids were lower levels of DHA and higher amounts of AdA, as shown in the fatty acid profiles of PI, phosphatidylserine (PS), total GPE and total GPC (Tables 6–8). The resulting higher n-6/n-3 ratio in the total phospholipid and individual phospholipids in the *pcy* relative to DBA mouse kidneys (Fig. 1) are of some interest in view of the association of high dietary n-6/n-3 intakes with tumorigenicity (51).

The Δ4 desaturase activity (potential), as measured by the product/substrate levels, was found to be consistently lower across all phospholipids in *pcy* compared to DBA mouse kidneys (Fig. 2). A diminution of the Δ4-desaturase activity also has been implicated in Zellweger's syndrome (52,53), a disease characterized by an absence of peroxisomes in hepatocytes and renal proximal tubules (54,55). Zellweger's syndrome has been associated with PKD (56),

TABLE 3

Relative Amounts of Diacyl, Alkenylacyl and Alkylacyl Subclasses of GPE and GPC in DBA and *pcy* Kidneys[a]

	mol % of GPE or GPC	
	DBA	*pcy*
Diacyl GPE	72.1 ± 1.9	60.0 ± 4.0[b]
Alkenylacyl GPE	26.0 ± 2.3	37.3 ± 3.2[b]
Alkylacyl GPE	2.2 ± 0.7	2.7 ± 0.8
Diacyl GPC	82.7 ± 1.5	85.0 ± 1.8
Alkenylacyl GPC	<2.0	<2.0
Alkylacyl GPC	15.3 ± 2.1	13.6 ± 1.5
Alkenylacyl GPE/diacyl GPE ratio	0.36 ± 0.02	0.62 ± 0.05[b]

[a]Values represent means ± SE (n = 4). [b]Significantly different from DBA, $P < 0.01$.

TABLE 4

Fatty Acid Compositions of Total Phospholipid and TG in DBA and *pcy* Kidneys[a]

Fatty acid[b]	Total phospholipid		TG	
	DBA	*pcy*	DBA	*pcy*
16:0	21.9 ± 0.9	20.0 ± 0.9	18.9 ± 0.7	20.7 ± 0.8
16:1	0.7 ± 0.0	0.7 ± 0.7	3.0 ± 0.2	3.6 ± 0.2
18:0	15.6 ± 0.2	16.4 ± 0.1[c]	7.4 ± 0.2	7.8 ± 0.4
18:1	10.6 ± 0.2	11.7 ± 0.1[c]	37.9 ± 0.4	41.4 ± 2.1
18:2n-6	9.6 ± 0.3	8.6 ± 0.4	16.9 ± 1.1	16.1 ± 0.5
18:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
20:4n-6	19.6 ± 0.7	21.6 ± 0.8	1.8 ± 0.1	1.7 ± 0.5
22:4n-6	0.4 ± 0.1	1.0 ± 0.2[d]	0.3 ± 0.0	0.2 ± 0.1
22:5n-6	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.1[d]
22:5n-3	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
22:6n-3	10.8 ± 0.7	8.4 ± 0.3[d]	2.9 ± 0.4	2.1 ± 0.6
24:0	1.9 ± 0.2	1.6 ± 0.1	trace	trace
24:1	0.8 ± 0.1	1.0 ± 0.1	trace	trace

[a]Values given as mol % of total fatty acids (means ± SE, n = 4).
[b]Other minor fatty acids are omitted from the table.
[c]Significantly different from DBA, $P < 0.01$.
[d]Significantly different from DBA, $P < 0.05$.

TABLE 5

Fatty Acid Compositions of Total Phospholipid and TG in DBA and *pcy* Serum[a]

Fatty acid[b]	Total phospholipid		TG	
	DBA	*pcy*	DBA	*pcy*
16:0	26.8 ± 0.8	25.1 ± 0.5	24.9 ± 0.9	24.1 ± 0.2
16:1	0.7 ± 0.0	0.5 ± 0.1[c]	3.5 ± 0.2	3.2 ± 0.1
18:0	20.2 ± 0.9	20.5 ± 0.5	2.9 ± 0.2	3.0 ± 0.1
18:1	10.1 ± 0.4	8.8 ± 0.1[c]	37.0 ± 0.7	34.7 ± 0.6[c]
18:2n-6	13.6 ± 0.5	15.1 ± 0.2[c]	19.6 ± 0.4	20.5 ± 0.4
18:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.0	0.6 ± 0.0
20:4n-6	15.9 ± 0.5	17.6 ± 0.2[c]	3.4 ± 0.6	5.0 ± 0.6
22:4n-6	trace	trace	0.2 ± 0.0	0.2 ± 0.0
22:5n-6	0.4 ± 0.0	0.2 ± 0.0[c]	0.3 ± 0.0	0.3 ± 0.0
22:5n-3	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
22:6n-3	6.9 ± 0.2	6.7 ± 0.1	2.5 ± 0.2	3.5 ± 0.2[d]

[a]Values given as mol % of total fatty acids (means ± SE, n = 4).
[b]Other minor fatty acids are omitted from the table.
[c]Significantly different from DBA, $P < 0.05$.
[d]Significantly different from DBA, $P < 0.01$.

TABLE 6

Fatty Acid Compositions of PI and PS in DBA and *pcy* Kidneys[a]

Fatty acid[b]	PI		PS	
	DBA	*pcy*	DBA	*pcy*
16:0	5.3 ± 0.2	5.2 ± 0.1	9.6 ± 0.6	6.1 − 0.5[c]
18:0	39.4 ± 0.1	38.9 ± 0.3	36.9 ± 0.4	39.8 ± 0.6[d]
18:1	6.9 ± 0.2	7.3 ± 0.2	9.9 ± 0.2	14.8 ± 0.8[d]
18:2n-6	2.6 ± 0.2	2.5 ± 0.1	3.4 ± 0.2	3.1 ± 0.1
20:3n-6	1.6 ± 0.1	1.3 ± 0.1	0.5 ± 0.0	0.5 ± 0.0
20:4n-6	34.6 ± 0.2	35.6 ± 0.5	27.1 ± 0.8	21.4 ± 1.1[d]
22:4n-6	0.3 ± 0.0	0.8 ± 0.1[e]	1.1 ± 0.1	2.1 ± 0.4
22:5n-6	0.3 ± 0.0	0.3 ± 0.0	0.8 ± 0.1	0.4 ± 0.1[c]
22:5n-3	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.5 ± 0.1
22:6n-3	4.2 ± 0.2	2.9 ± 0.2[d]	5.0 ± 0.4	3.7 ± 0.2[c]

[a]Values given as mol % of total fatty acids (means ± SE, n = 4).
[b]Other minor fatty acids are omitted from the table.
[c]Significantly different from DBA, $P < 0.05$.
[d]Significantly different from DBA, $P < 0.01$.
[e]Significantly different from DBA, $P < 0.001$.

indicating that they may have some common lipid abnormalities. Recently, it has been reported that the Δ4 desaturase enzyme is not present in rat hepatocytes (57). The proposed pathway for conversion of 22:5n-3 to DHA is 22:5n-3 → 24:5n-3 → 24:6n-3 → DHA, with the last step being analogous to peroxisomal β-oxidation of long-chain fatty acids. If this pathway is present in murine renal cells, an abnormality in this pathway may also explain the higher 22:4n-6 plus 22:5n-3 to 22:5n-6 plus 22:6n-3 ratio in *pcy* mouse kidneys.

With respect to the potential relevance of the lower DHA levels in phospholipid to PKD, reduced levels of DHA have been reported in the lipids of a high metastatic cell line when compared to one of low metastatic potential (8) and in hepatoma cells compared to normal hepatocytes (4). Both the lower levels of renal DHA and the higher cholesterol/phospholipid ratio (Table 1) seen in *pcy* mice may result in a decrease in membrane fluidity, possibly altering membrane-mediated events. In a mouse model of essential fatty acid deficiency, Sun and Sun (58) documented a decrease in brain DHA level and an increase in Na^+K^+ATPase activity. Surette and colleagues (59) reported that dietary DHA increased mouse microsomal DHA content and decreased the specific activity of Na^+K^+ATPase by 48%. In this regard, altered DHA and cholesterol/phospholipid levels seen herein may be associated with the elevation of the activity of this membrane-bound enzyme in PKD (60).

The higher level of AdA in the kidney phospholipid of the *pcy* mouse kidney relative to the DBA is of interest because AdA can be metabolized by the kidney *via* the cyclooxygenase enzyme to produce dihomo-prostaglandins and dihomo-thromboxane, unlike other tissues enriched in AdA, such as the adrenal glands, brain and testis (61). Furthermore, the production of these AdA-derived eicosanoids was found to be augmented in the hydronephrotic kidney (61). The potential implications of increased levels of AdA in the kidney phospholipid in relation to the formation/function of these unique eicosanoids in PKD remains to be evaluated.

Fatty acid analysis of sphingomyelin (SPH) revealed only modest differences between the *pcy* and DBA mouse kidneys; these included a higher level of arachidic acid (20:0) in the former group (Table 9).

Unlike the other kidney phospholipids, DPG exhibited higher relative levels of DHA in the *pcy* kidneys (Table 9) and a lower n-6/n-3 fatty acid ratio (Fig. 1). As a mitochondrial phospholipid, these fatty acid alterations in DPG, as well as the lower levels of DPG in *pcy* (Table 2), may be associated with possible mitochondrial changes in PKD. In this regard, renal mitochondria are reduced compared to hepatic mitochondria in cisplatin-induced nephrotoxicity (62). Cisplatin administration has been shown to induce renal cyst growth (63). The inability of renal tissue

TABLE 7

Fatty Acid Compositions of Total GPE and the Diacyl and Alkenylacyl Subclasses of GPE in DBA and *pcy* Kidneys[a]

Fatty acid[c]	Total GPE		Diacyl GPE		Alkenylacyl GPE[b]	
	DBA	*pcy*	DBA	*pcy*	DBA	*pcy*
16:0	11.1 ± 0.2	10.3 ± 0.6	11.3 ± 0.4	9.4 ± 0.2[d]	2.3 ± 0.7	1.8 ± 0.4
18:0	22.6 ± 0.1	21.7 ± 1.0	26.2 ± 0.3	26.8 ± 0.3	1.5 ± 0.2	1.7 ± 0.1
18:1	10.9 ± 0.2	10.7 ± 0.1	12.6 ± 0.3	12.8 ± 0.2	5.2 ± 0.9	4.7 ± 0.5
18:2n-6	3.6 ± 0.1	3.2 ± 0.1[e]	4.0 ± 0.2	3.9 ± 0.1	2.5 ± 0.3	1.7 ± 0.2
20:4n-6	29.4 ± 0.3	29.8 ± 1.0	33.5 ± 0.5	33.8 ± 0.4	45.4 ± 1.0	56.5 ± 2.0[d]
22:4n-6	0.6 ± 0.0	1.3 ± 0.2[d]	0.4 ± 0.0	1.2 ± 0.2[e]	1.9 ± 0.0	3.7 ± 0.3[d]
22:5n-6	0.7 ± 0.1	0.5 ± 0.0[e]	0.8 ± 0.1	0.7 ± 0.0	0.9 ± 0.1	0.8 ± 0.1
22:5n-3	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.8 ± 0.0	0.9 ± 0.1
22:6n-3	11.2 ± 0.3	8.2 ± 0.5[d]	8.6 ± 0.6	8.7 ± 0.3	35.4 ± 1.4	24.9 ± 2.0[d]

[a]Values given as mol % of total fatty acids (means ± SE, n = 4).

[b]Fatty acids in the *sn*-2 position.

[c]Other minor fatty acids are omitted from the table.

[d]Significantly different from DBA, $P < 0.01$.

[e]Significantly different from DBA, $P < 0.05$.

TABLE 8

Fatty Acid Compositions of Total GPC and the Diacyl and Alkylacyl Subclasses of GPC in DBA and *pcy* Kidneys[a]

Fatty acid[c]	Total GPC		Diacyl GPC		Alkylacyl GPC[b]	
	DBA	*pcy*	DBA	*pcy*	DBA	*pcy*
16:0	35.7 ± 1.2	30.9 ± 0.7[d]	35.8 ± 0.6	32.5 ± 0.2[d]	6.5 ± 0.5	9.7 ± 0.9[d]
18:0	9.5 ± 0.2	12.3 ± 0.3[e]	10.8 ± 0.3	13.6 ± 0.5[f]	0.3 ± 0.1	0.7 ± 0.3
18:1	13.5 ± 0.3	15.6 ± 0.3[f]	15.1 ± 0.2	17.0 ± 0.4[f]	2.1 ± 0.2	3.1 ± 0.2[f]
18:2n-6	8.9 ± 0.4	9.1 ± 0.1	9.7 ± 0.4	9.8 ± 0.1	2.1 ± 0.1	2.6 ± 0.1[d]
20:4n-6	13.5 ± 0.6	16.3 ± 0.9[d]	14.4 ± 0.2	15.9 ± 0.2[f]	13.3 ± 1.1	23.4 ± 2.1[f]
22:4n-6	0.2 ± 0.0	0.6 ± 0.1[d]	0.3 ± 0.0	0.5 ± 0.1[d]	0.3 ± 0.0	1.0 ± 0.2[d]
22:5n-6	0.4 ± 0.0	0.2 ± 0.0[d]	0.4 ± 0.0	0.2 ± 0.0[f]	0.7 ± 0.1	0.5 ± 0.1
22:5n-3	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	1.4 ± 0.1	1.2 ± 0.1
22:6n-3	14.0 ± 0.8	9.4 ± 0.8[f]	9.8 ± 0.3	6.2 ± 0.8[f]	66.7 ± 1.6	51.1 ± 3.4[f]

[a]Values given as mol % of total fatty acids (means ± SE, n = 4).

[b]Fatty acids in the *sn*-2 position.

[c]Other minor fatty acids are omitted from the table.

[d]Significantly different from DBA, $P < 0.05$.

[e]Significantly different from DBA, $P < 0.001$.

[f]Significantly different from DBA, $P < 0.01$.

to replace damaged mitochondria possibly may explain why the kidneys (and not livers) are susceptible to cyst formation in the *pcy* mouse. The lower level of DPG in the *pcy* kidneys (Table 2) was of some interest as well, because the maintenance of mitochondrial carnitine palmitoyl transferase activity in mammalian membranes requires the presence of DPG (64).

Interestingly, separation of the total GPE into the diacyl and alkenylacyl subclasses (Table 7), revealed that the lower GPE level of DHA in the *pcy* kidney was associated with the alkenylacyl GPE (lower by 30%) and not the diacyl GPE (which exhibited no difference in DHA content). The biochemical basis for this phenomenon (including the availability of DHA-containing precursors or enzyme specificities leading to alkenylacyl and diacyl GPE biosynthesis and breakdown) and significance to PKD remain to be investigated. The higher level of AdA seen in total kidney phospholipid (Table 4) was maintained in the total GPE, diacyl GPE and alkenylacyl GPE (Table 7). The lower level of DHA and higher levels of AdA, as well as arachidonic acid, were consistent in total GPC and the two subclasses, diacyl GPC and alkylacyl GPC (Table 8). An evaluation of platelet-activating factor involvement in PKD, *via* its formation from renal alkylacyl GPC, remains to be studied. Platelet-activating factor synthesis has been implicated recently in a number of renal pathophysiologies (65).

In conclusion, mouse *pcy* kidneys are characterized by several lipid abnormalities often seen in neoplastic cells, such as a higher cholesterol/phospholipid and GPC/GPE

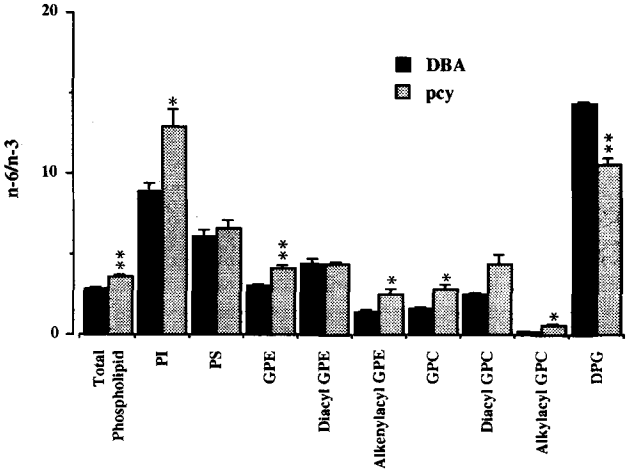

FIG. 1. Renal n-6/n-3 levels in DBA and *pcy* mice. *Significantly different from DBA, $P < 0.05$. **Significantly different from DBA, $P < 0.01$.

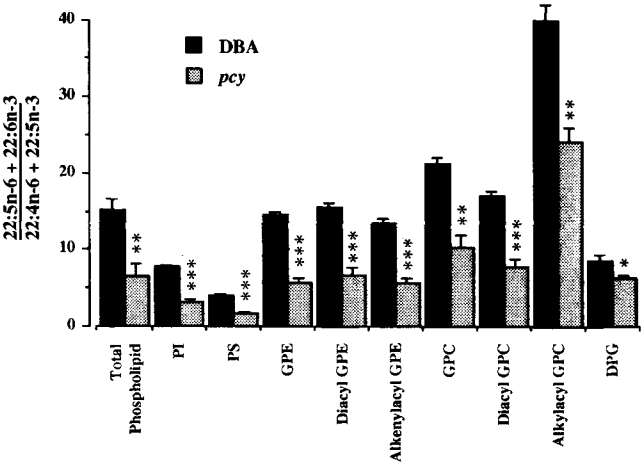

FIG. 2. Total potential Δ4 desaturase activity as assessed by product/substrate ratios in DBA and *pcy* mouse kidneys. *Significantly different from DBA, $P < 0.05$. **Significantly different from DBA, $P < 0.01$. ***Significantly different from DBA, $P < 0.001$.

ratios, lower DHA levels, and relative enrichment of ether phospholipid (notably alkenylacyl GPE). The higher cholesterol/phospholipid and GPC/GPE ratios and lower DHA levels may alter membrane fluidity, thereby altering membrane functions related to cell growth, such as receptor activation, signal transduction, ion transport and enzyme activities. The possible influence of nutritional modification (*e.g.*, dietary DHA supplementation) on the lipid abnormalities exhibited in this model of PKD remains to be evaluated.

ACKNOWLEDGMENTS

This work was supported by a research grant from the Natural Sciences and Engineering Research Council of Canada.

TABLE 9

Fatty Acid Compositions of SPH and DPG in DBA and *pcy* Kidneys[a]

Fatty acid[b]	SPH		DPG	
	DBA	*pcy*	DBA	*pcy*
16:0	37.9 ± 0.7	34.7 ± 2.9	2.7 ± 0.1	3.6 ± 0.2[c]
18:0	3.1 ± 0.1	4.2 ± 0.3[d]	1.1 ± 0.0	1.6 ± 0.2[c]
18:1	2.1 ± 0.0	2.3 ± 0.2	19.8 ± 0.3	21.5 ± 0.6
18:2n-6	0.4 ± 0.0	0.4 ± 0.0	57.9 ± 0.3	52.3 ± 1.3[d]
20:0	2.9 ± 0.3	4.2 ± 0.1[d]	0.1 ± 0.0	0.2 ± 0.0
20:2n-6	0.5 ± 0.2	0.1 ± 0.0	2.8 ± 0.1	3.1 ± 0.1
20:3n-6	0.4 ± 0.0	0.5 ± 0.0[c]	1.6 ± 0.1	1.2 ± 0.1[d]
20:4n-6	0.4 ± 0.0	0.5 ± 0.1	2.6 ± 0.1	3.7 ± 0.2[d]
22:0	13.4 ± 0.3	16.0 ± 1.1	0.2 ± 0.0	0.4 ± 0.0[c]
22:1	0.5 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.4 ± 0.0
22:4n-6	trace	trace	0.2 ± 0.0	0.6 ± 0.0[e]
22:5n-6	trace	trace	0.4 ± 0.0	0.7 ± 0.1[d]
22:5n-3	trace	trace	0.2 ± 0.0	0.3 ± 0.0
22:6n-3	0.3 ± 0.0	0.3 ± 0.0	3.4 ± 0.1	4.7 ± 0.3[e]
23:0	2.7 ± 0.1	2.4 ± 0.4	trace	trace
24:0	20.1 ± 0.4	17.5 ± 1.7	0.3 ± 0.1	0.2 ± 0.0
24:1	9.4 ± 0.3	9.8 ± 0.5	trace	trace

[a] Values given as mol % of total fatty acids (means ± SE, n = 4).
[b] Other minor fatty acidds are omitted from the table.
[c] Significantly different from DBA, $P < 0.05$.
[d] Significantly different from DBA, $P < 0.01$.
[e] Significantly different from DBA, $P < 0.001$.

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Characterization of Triacylglycerols in the Seeds of *Aquilegia vulgaris* by Chromatographic and Mass Spectrometric Methods

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A combination of analytical techniques is generally necessary to properly characterize complex lipid materials. Chromatographic separation in conjunction with spectroscopic characterization was utilized for the analysis of the triacylglycerols in the seeds of *Aquilegia vulgaris*. Reversed-phase high-performance liquid chromatography (HPLC), micropacked argentation supercritical fluid chromatography (SFC), and combinations of the two techniques were used. The fatty acid profile was determined by gas chromatography/mass spectrometry of the picolinyl esters and by gas chromatography/flame-ionization detection of the methyl esters. The major components were also identified by direct inlet mass spectrometry. The excellent selectivity of packed fused silica argentation SFC for the separation of triacylglycerols was demonstrated.

Lipids 27, 436–441 (1992).

Essential fatty acid (EFA) deficiency in mammals caused by an unbalanced diet can lead to conditions such as scaly dermatitis, interactable eczema and excessive transdermal loss of body water (1–3). Dietary supplementation of essential fatty acids, mainly linoleic and α-linolenic acids, normally reverses these conditions. Columbinic acid (Cb), 6*c*,9*c*,13*t*-18:3, has been found in abundance in the seeds of the Sierra Meadowrue *(Thalictrum polycarpum)* (4) and in the seeds of the Columbine *(Aquilegia vulgaris)* (5,6). This fatty acid exhibits EFA properties similar to linoleic acid and α-linolenic acid in mammals. This indicates that columbinic acid undergoes metabolic conversion by utilizing enzyme systems that generally metabolize arachidonic acid (7). It has also been shown that the major lipoxygenase product of columbinic acid, 13-hydroxyoctadecatrienoic acid, can reverse scaly dermatitis in EFA deficient rats (7).

The fatty acid profile of the seed oil of the *Aquilegia vulgaris* has been determined (8), but the molecular species pattern of the triacylglycerols, which has important physicochemical and bioavailability implications, has not yet been investigated.

Triacylglycerols have been extensively characterized by gas chromatography (GC) and high-performance liquid chromatography (HPLC) (9,10). The applicability of GC is, however, limited by the thermal stability of the triacylglycerols. Thermal degradation and/or polymerization of polyunsaturated triacylglycerols takes place at the temperatures necessary for their elution (9–13).

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Abbreviations: C, 9*c*,13*t*-18:2; Cb, columbinic acid; EFA, essential fatty acids; FID, flame-ionization detector; GC, gas chromatography; HPLC, high-performance liquid chromatography; L, linoleic acid; MS, mass spectrometry; O, oleic acid; P, palmitic acid; +EI, positive electron impact; S, stearic acid; SFC, supercritical fluid chromatography; TG, triacylglycerol; UV, ultraviolet.

Excellent separations of triacylglycerol species have been obtained by HPLC in the reversed phase mode and on columns impregnated with silver nitrate (10). It was demonstrated by Christie and coworkers (14–17) that highly stable argentation columns can be prepared by silver nitrate impregnation of columns packed with a silica-based cation exchanger. The separation of molecular species is, however, oftentimes incomplete, and quantification can become difficult in such cases. The separation can be improved by a two-step procedure, *e.g.* by HPLC/GC (18) or HPLC/HPLC (19).

Micropacked argentation columns have recently been utilized for the separation of triacylglycerols using supercritical fluids as the mobile phase (20,21). It was also discovered that the Ag^+-modified, silica-based cation exchanger is very stable at elevated temperatures. This significantly increases the possibilities to optimize separation conditions, and temperature can thus be used to control silver/olefin complex formation. Further, microcolumns prepared from fused silica capillary tubing provide rapid and efficient heat transfer, which is a requirement for successful temperature programming.

Detection may be a problem when silica-based cation exchangers are being used as column packing material in microcolumn supercritical fluid chromatography (SFC). A polar modifier must be added to the mobile phase in order to elute triacylglycerols. The presence of such an additive precludes the use of flame-ionization detection; thus detection had to be by ultraviolet (UV) detector in the present work. This detector has a widely varying response for triacylglycerols dependent on the degree of unsaturation of the constituent fatty acyl chains.

In the present study, the triacylglycerol molecular species of the seed oil of *Aquilegia vulgaris* have been characterized by reversed phase HPLC, micropacked argentation SFC, direct inlet mass spectrometry (MS), and GC/MS of the picolinyl esters of the constituent fatty acids.

EXPERIMENTAL PROCEDURES

Materials. Seeds from *Aquilegia vulgaris* (Fröcentralen, Landskrona, Sweden) were cold-pressed to obtain the oil, which was analyzed without further purification.

Reversed phase HPLC. Fractionation of the triacylglycerols of the seed oil into molecular species was performed by HPLC at room temperature, utilizing a 250 mm × 10 mm column packed with Lichrospher 100, RP 18, 5 μm (Merck, Darmstadt, Germany). The seed oil was dissolved (50 mg/mL) in ethanol/isooctane (3:1, vol/vol). A volume of 200 μL was injected into the chromatograph with HPLC grade, degassed methanol/acetone (1:1, vol/vol) as the mobile phase. The solvent flow, which initially was set to 2.5 mL/min, was increased to 4 mL/min after 50 min. The total run time was 60 min. Detection was by means of a light-scattering detector (ACS, Macclesfield, United

Kingdom) operated at a dispersion pressure of 22 psi and a nebulizer temperature of 50°C. Between the outlet of the column and the detector, a split valve (1:4) was installed. Fractions representing molecular species were collected from four consecutive runs and the corresponding fractions subsequently combined. An aliquot of each fraction was derivatized to the corresponding methyl esters and analyzed by gas chromatography to determine the fatty acid composition.

Analytical HPLC was done on a 150 mm × 4.6 mm column packed with Ultraspher 100, ODS, 5 μm (Beckman Instruments, San Ramon, CA). The column was held at 25°C throughout the analysis. The solvent delivery system consisted of two Shimadzu (Kyoto, Japan) LC-6A HPLC pumps and a Shimadzu SCL-6A gradient control unit. A binary solvent gradient from (A) acetonitrile to (B) acetonitrile/ethanol/isooctane (2:2:1, vol/vol/vol) was applied over 60 min at a constant flow of 1 mL/min. Detection was with a light-scattering detector (Cunow, Cergy St. Christophe, France) operated at a dispersion pressure of 30 psi and a nebulizer temperature of 60°C.

Fatty acid methyl esters. Fatty acid methyl esters were analyzed as described earlier (22) utilizing an alkali methanolysis derivatization procedure in conjunction with a GC separation method. However, in the present work, the analytes were separated on a DB-Wax column, 30 m × 0.25 mm, film thickness 0.25 μm (J & W, Folsom, CA). Helium was used as carrier gas. On-column injection was at 130°C, after 2 min, programmed at 50°C/min to 150°C and then programmed at 3.3°C/min.

GC/MS of picolinyl esters. Picolinyl esters were prepared according to Christie and coworkers (23–25). The esters were analyzed by GC/MS using an ion trap detector (Varian ITS, Sunnyvale, CA) connected to a Varian 3400 gas chromatograph. The column used was a Supelcowax 10, 30 m × 0.25 mm, film thickness 0.25 μm (Supelco, Bellefonte, PA). Helium was used as carrier gas. On column injection was at 80°C, after 1 min, programmed at 50°C/min to 200°C, then programmed at 3.6°C/min. Fraction A was derivatized and analyzed.

Micropacked argentation SFC. The chromatographic system consisted of a Lee Scientific (Salt Lake City, UT) 600 Series SFC and μ-UV detector; detection was at 210 nm. Columns were prepared from fused silica capillary tubing, 330 mm × 250 μm i.d. and 430 μm o.d. (Polymicro Technologies, Phoenix, AZ). All columns were packed with Nucleosil 5 SA (Macherey Nagel, Düren, Germany). After packing, the columns were rinsed with 400 μL of 0.1 M silver nitrate (21).

The mobile phase consisted of carbon dioxide/acetonitrile/isopropanol (92.8:6.5:0.7, by mol%). Critical parameters for this mixture have been reported earlier (20). The mobile phase mixture was prepared in the SFC pump as described previously (26). Mobile phase velocity was 3.5 mm/s. Seed oil of *Aquilegia vulgaris* and fractions thereof, obtained from HPLC, were analyzed. Solutes were dissolved in HPLC-grade pentane at concentrations of 30 mg/mL. The injector was equipped with an internal loop, 200 nL, and injection was performed using a timed split of 0.2 s. Furthermore, a split ratio of 1:1 was applied. Using these conditions, *ca.* 60 nL was allowed to enter the column.

Direct inlet mass spectroscopy. Mass spectrometric analysis of triacylglycerols was carried out on a Triple Stage Quadrupol (TSQ) 4600 (Finnigan Mat, San José, CA) equipped with a water-cooled TIC controlled solid inlet system (Finnigan Mat, Bremen, Germany). The ion source temperature was 150°C. Ionization energy was 70 eV. Direct inlet temperature was programmed from 50°C to 450°C at 10°/s. The solvent was evaporated prior to introduction into the MS.

RESULTS AND DISCUSSION

In this study, the triacylglycerols (TG) of the seeds of *Aquilegia vulgaris* were characterized in terms of molecular species composition and fatty acid profile. This was accomplished by (i) a traditional approach utilizing preparative reversed phase HPLC to fractionate groups of molecular species similar in chain length and unsaturation, followed by subsequent fatty acid determination of each collected fraction, and (ii) the novel technique of micropacked argentation-SFC by which separation is achieved mainly according to degree of unsaturation.

The analytical HPLC chromatogram of the triacylglycerols is shown in Figure 1, and the preparative chromatogram in Figure 2. Note that the acyl groups are given in an arbitrary sequence and do not denote any specific position at the glycerol moiety. Fractions, as indicated in Figure 2, were collected for further analysis. All the

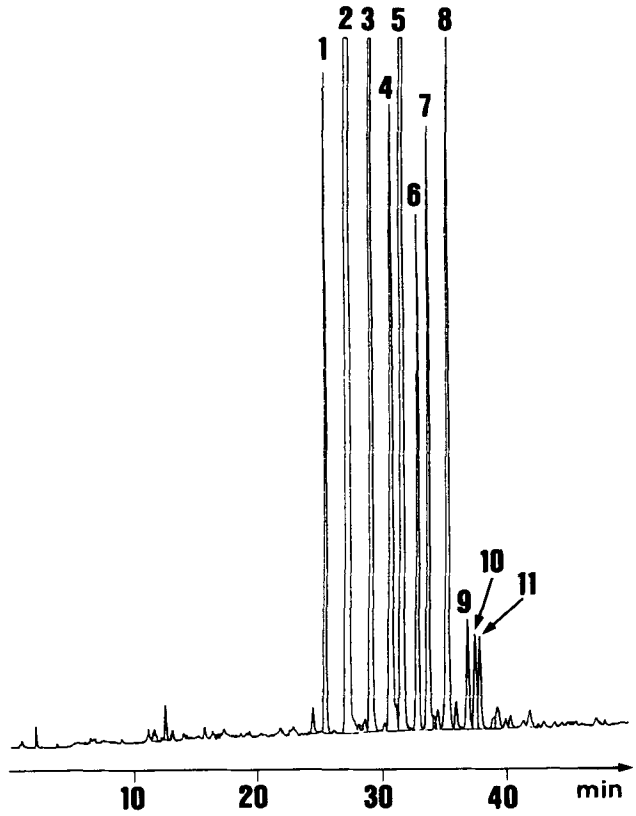


FIG. 1. Analytical reversed phase high-performance liquid chromatogram of the triacylglycerols present in the seed oil of *Aquilegia vulgaris*. Column: Ultrasphere 100, ODS, 5 μm (150 × 4.6 mm). Conditions: see text, reversed-phase HPLC. Detection: Light-scattering. Peaks: 1, CbCbCb; 2, CbCbL; 3, CbLL; 4, CbCbO; 5, CbCbP; 6, CbLO; 7, CbLP; 8, CbCbS; 9, CbOO; 10, CbLS; 11, CbOP.

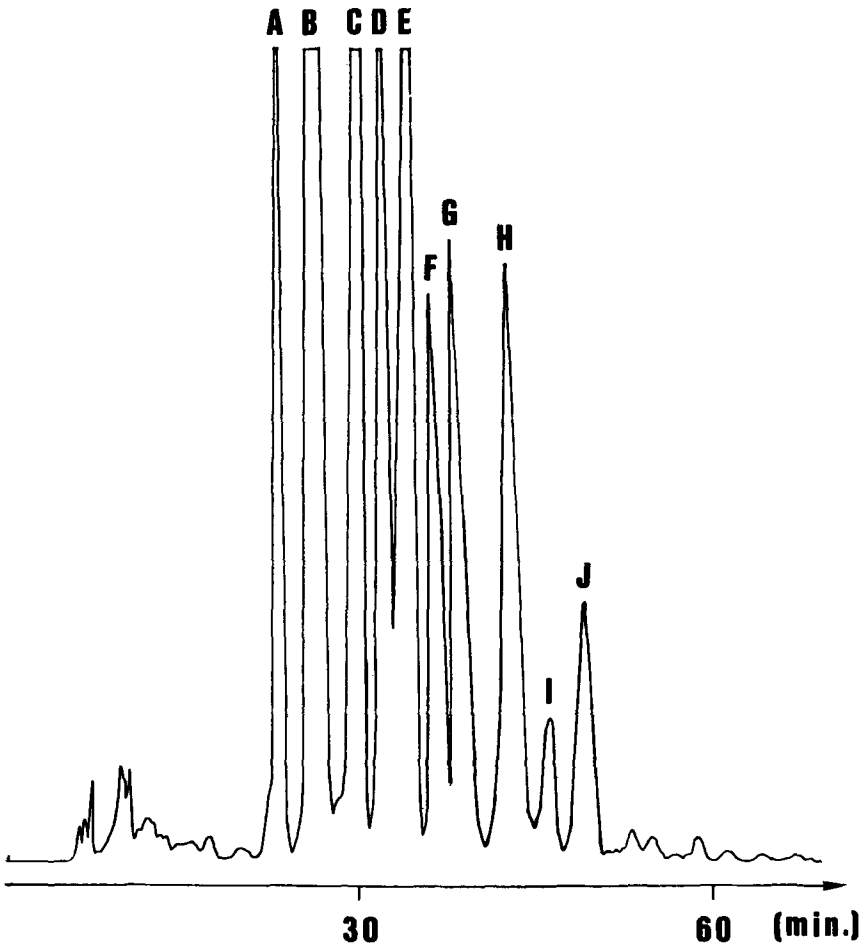


FIG. 2. Preparative reversed-phase high-performance liquid chromatogram of the triacylglycerols present in the seed oil of *Aquilegia vulgaris*. Column: Lichrosphere 100, RP 18, 5 μm (250 × 10 mm). Conditions: see text, reversed-phase HPLC. The main components of each fraction are given in Table 1.

fractions were thus analyzed by SFC/UV; the purpose of this was to control the peak purity and to establish the elution order. Some of the fractions were derivatized to picolinyl esters which made it possible to determine the position of the double bonds in the acids by GC/MS (23–25). A mass spectrum of the picolinyl esters of columbinic acid is shown in Figure 3. The fatty acid picolinyl ester was obtained by hydrolysis of Fraction A. The peaks at $m/z = 218$, 258 and 298 indicate the positions of the double bonds (see Scheme 1).

The fatty acid profiles of the oil and its different HPLC fractions were quantitatively analyzed by means of GC/FID (flame-ionization detector) of the methyl esters derived from TG. The results are given in Table 1. It can be noted that there is some overlap between the different fractions. The composition of the oil was in good agreement with published data (8,27).

The fractions were further analyzed by direct inlet MS in the positive electron impact mode (+EI). This technique provides information concerning the chain length and the number of double bonds (28–32). The fragmentation of

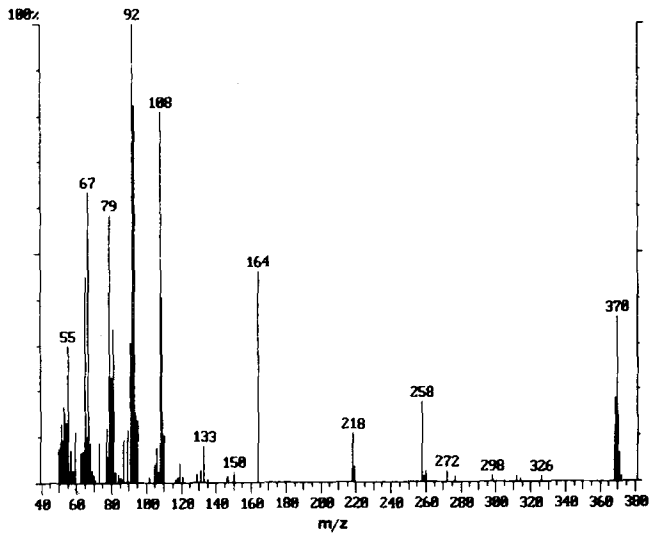
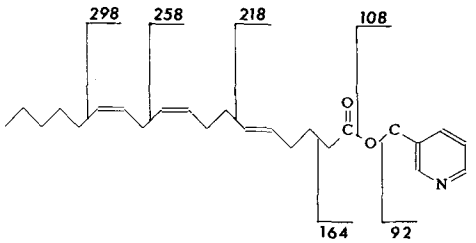


FIG. 3. Mass spectrum of the picolinyl esters of columbinic acid. Conditions: see text, GC/MS of picolinyl esters.



SCHEME 1

triacylglycerols has been extensively investigated (28–32). Characteristic ions corresponding to M^+, $[M - RCOO]^+$, $[RCO + 128]^+$, $[RCO + 74]^+$ and $[RCO]^+$ are being formed. R represents the aliphatic chain of the constituent fatty acids.

The results are summarized in Table 2. The main components of the fractions have been definitively identified from the ions $[RCO]^+$, $[RCO - 1]^+$ and $[M - OCOR]^+$. Molecular weight is indicated by $[M]^+$. Under the ionization conditions applied, saturated TG give relatively small M^+ ions (32); the intensity of M^+ is, however, increased with increasing degree of unsaturation in the TG. Considering the stability of the ions $[RCO + 128]$, saturated fatty acids give the highest intensities, and the intensities of unsaturated fatty acids are decreasing with an increasing degree of unsaturation. A mass spectrum of Fraction C is given in Figure 4.

Separation of the oil by microcolumn argentation SFC is shown in Figure 5. Elution in HPLC as shown in Figures 1 and 2 is obtained according to partition number, while separation, as performed in Figure 5, depends on the number and position of double bonds. Furthermore, some separation according to chain length is indicated in Figure 5, *e.g.*, CbCbP (where P is palmitic acid) from CbCbS (where S is stearic acid). Oil from *Aquilegia vulgaris* has a relatively simple composition, and all major components could be separated on the SFC column. The peaks in Figure 5 have been identified with some different degree of certainty. Identification of the major peaks was by direct inlet MS of the fractions (Table 2) and injection of the same fractions onto the micro SFC system. Trilinolein was tentatively identified on the basis of the fatty acid profile of Fraction E and the retention time of a standard. The separation of Fraction E on microcolumn argentation SFC is shown in Figure 6. LLL (where L is linoleic acid) was recently detected also in meadowfoam oil (25,27). Further, the presence of LPP, LOP (where O is oleic acid), LLP, LLS, LOO and LLO in the oil was indicated by minor peaks in Figure 5 having the same retention as observed for these TG under identical SFC conditions, *e.g.* in soybean oil (21). Finally, TG containing the acid 9*c*,13*t*-18:2 (C) may be indicated in Figure 5. Tentative identification was based on fatty acid profiles (Table 1) and retention times.

In reversed-phase HPLC, TG having identical carbon numbers and numbers of double bonds are eluted according to the position of the double bonds (10). The highest content of the acid 9*c*,13*t*-18:2 was observed in Fraction D (Table 1). It seems that 9*c*,13*t*-18:2 is derived from species such as 9*c*,13*t*-18:2, 9*c*,13*t*-18:2, and 6*c*,9*c*,13*t*-18:3. The retention of this species in reversed-phase HPLC is higher than for CbLL, and therefore, it has been collected in Fraction D rather than in Fraction C. Separation of Fraction D on argentation SFC revealed a small peak right after the elution of CbCbO. In argentation chromatography, *trans*-isomers migrate ahead of corresponding *cis*-isomers (9). It may thus be speculated that the peak preceding CbLL consists of 9*c*,13*t*-18:2, 9*c*,13*t*-18:2, and 6*c*,9*c*,13*t*-18:3. Similarly, Peaks 2 and 9 may contain the species 9*c*,13*t*-18:2, 6*c*,9*c*,13*t*-18:3, 16:0, and 9*c*,13*t*-18:2, 6*c*,13*t*-18:3, 9*c*-18:1, respectively.

This study has shown that the seeds of *A. vulgaris* are a rich source of columbinic acid and that this acid is incorporated in the majority of molecular species of triacylglycerols. The biological implications of this would merit further investigations.

With the recent introduction of micropacked argentation-SFC, an alternative route of lipid separation has

TABLE 1

Fatty Acid Composition (%)[a] of *Aquilegia vulgaris* Seed Oil and of Fractions Obtained by Reversed-Phase HPLC

	Total composition (% w/w)	Fraction									
		A	B	C	D	E	F	G	H	I	J
16:0	7.6	0.4	—	0.5	2.4	28.5	2.4	25.4	6.0	2.9	15.4
18:0	2.7	0.3	—	—	—	—	—	0.3	20.9	1.5	15.7
20:0	0.1	—	—	—	0.5	—	—	0.9	—	0.6	0.1
9*c*-18:1	5.1	0.4	—	—	16.0	0.8	26.2	3.5	2.8	52.9	15.7
7*c*-18:1	0.3	—	—	—	3.8	0.1	1.7	0.4	0.3	2.0	—
11*c*-20:1	0.1	—	—	—	0.1	—	—	—	—	—	—
9*c*,13*t*-18:2	1.4	0.3	—	0.5	7.8	0.2	1.3	2.8	3.6	2.4	1.6
6*c*,9*c*-18:2	25.3	3.7	32.6	58.2	11.2	3.6	32.2	29.4	9.8	7.0	17.2
6*c*,9*c*,13*t*-18:3	56.1	89.9	66.8	36.7	57.7	66.2	33.3	34.7	56.4	30.7	33.5
3*c*,6*c*,9*c*-18:3	0.1	1.1	—	—	—	—	0.2	—	—	—	—
3*c*,6*c*,9*c*,12*c*-18:4	0.2	1.7	—	0.7	—	—	0.2	—	—	—	—
Unidentified	1.0	2.2	0.6	3.4	0.5	0.6	2.5	2.6	0.2	—	0.8
Principal molecular species		CbCbCb	CbCbL	CbLL	CbCbO	CbCbP	CbLO	CbLP	CbCbS	CbOO	CbLS + CbOP
Amount (area %)[b]		3.0	53.8	8.3	5.1	15.3	2.9	3.6	4.6	0.7	0.7
Standard deviation (n = 4)		0.1	0.6	0.1	0.1	0.1	0.1	0.2	0.2	0.04	0.1

[a]GC/flame-ionization detection. [b]HPLC/light-scattering detection; peak areas were not corrected for non-linearity.

TABLE 2

Proposed Molecular Species of Triacylglycerols and Observed MS Fragmentation

Fraction	TC/DB[a]	FA res.[b]	*m/z* of fragment ions					
			$[M]^+$	$[M - RCOO]^+$	$[RCO + 128]^+$	$[RCO + 74]^+$	$[RCO]^+$	$[RCO - 1]^+$
A	54:9	Cb	872	595	389	335		260
		Cb						260
		Cb						260
B	54:8	L	874	597	391	337		262
		Cb						260
		Cb				335		260
C	54:7	L	876	599	391	337		262
		Cb				335		260
		L						262
D	54:7	O	876	599	393	339		264
		Cb				335		260
		Cb						260
E	52:6	P	850	573	367	313	239	
		Cb						260
		Cb						260
F	54:6	O	878	601	393	339		264
		Cb				335		260
		L				337		262
G	52:5	P	852	575	367	313	239	
		Cb						260
		L						262
H	54:6	S	878	601	395	341	267	
		Cb						260
		Cb						260
I	54:5	O	880	603	393	339		264
		Cb						260
		O						264
J	54:5	S	880	603	395	341	267	
		Cb				335		260
		L				337		262
	52:4	P	854	557	367	313	239	
		Cb						260
		O						264

[a]TC, the number of total acyl carbons; DB, the number of double bonds. [b]FA res., acyl residue.

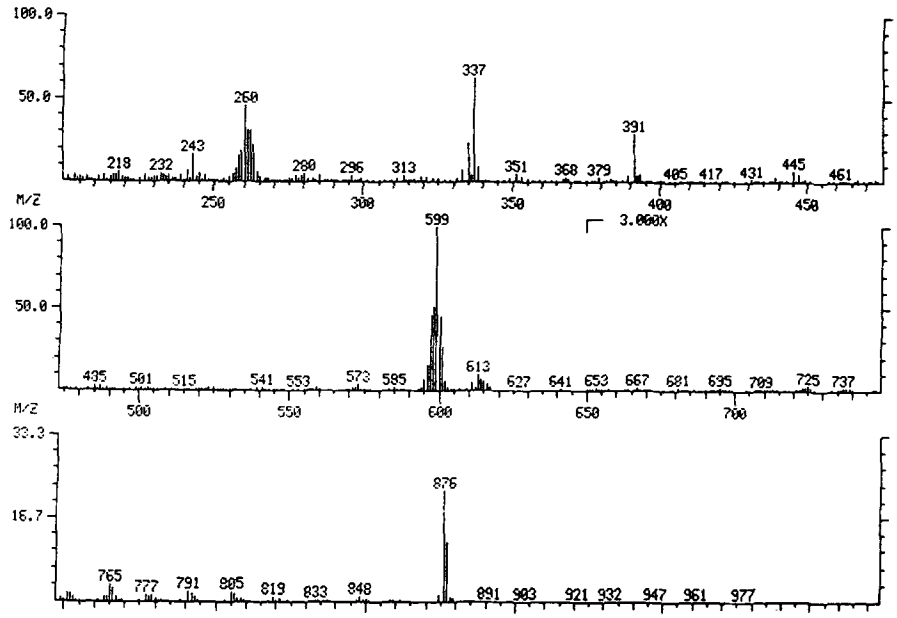


FIG. 4. Mass spectrum of Fraction C, Figure 2. Conditions: see text; direct inlet mass spectroscopy.

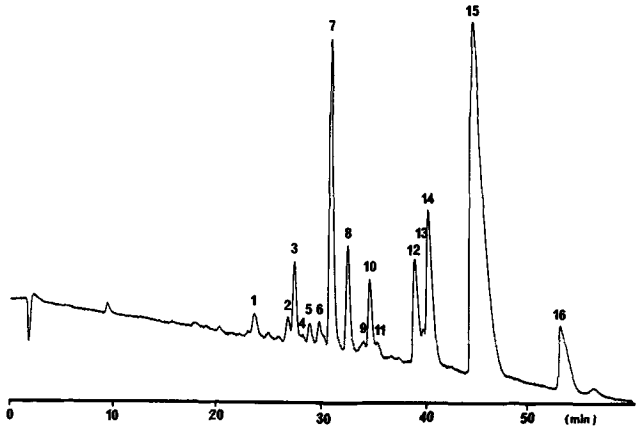


FIG. 5. Supercritical fluid chromatogram with UV detection at 210 nm of the triacylglycerols present in the seed oil from *Aquilegia vulgaris*. Column: fused silica, 330 mm × 250 μm, packed with Nucleosil 5 SA and impregnated with $AgNO_3$. Conditions: injection at 115°C and 260 atm, after 2 min, programmed at −0.5°/min and 1 atm/min to 85°C and 320 atm. Mobile phase: carbon dioxide/acetonitrile/isopropanol, 92.8:6.5:0.7, mol%. Peaks: 1, CbOP; 2, CCbP; 3, CbLP; 4, CCbS; 5, CbLS; 6, CbOO; 7, CbCbP; 8, CbCbS; 9, CCbO; 10, CbLO; 11, LLL; 12, CbCbO; 13, CCCb; 14, CbLL; 15, CbCbL; 16, CbCbCb.

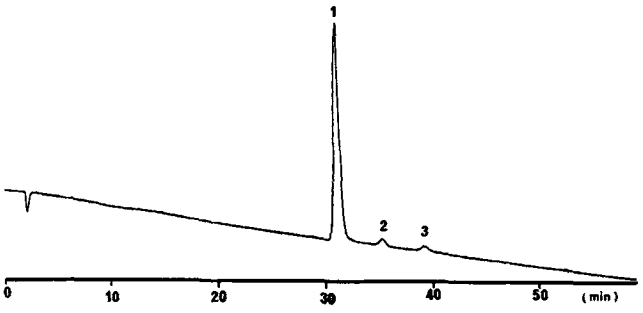


FIG. 6. Supercritical fluid chromatogram with UV detection of the triacylglycerols present in Fraction E. Column and conditions as in Figure 5. Peaks: 1, CbCbP; 2, LLL; 3, CbCbO.

emerged. The solubility properties of the mobile phase, *i.e.* the supercritical medium, places this type of chromatography in an interesting experimental domain. Even though the full potential of micropacked argentation-SFC is yet to be exploited, it has proven to be a worthy addition to the family of lipid analytical methods.

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Autoxidation of Polyunsaturated Triacylglycerols. IV. Volatile Decomposition Products from Triacylglycerols Containing Linoleate and Linolenate[1]

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Trilinoleoylglycerol (LLL), trilinolenoylglycerol (LnLnLn) and four synthetic triacylglycerols were autoxidized and the volatile products were investigated to determine the effect of fatty acid glyceride position on the mechanism of hydroperoxide decomposition. Capillary gas chromatography provided a sensitive method to follow the volatile oxidation products of mixtures of LLL and LnLnLn and of synthetic triacylglycerols containing linoleate and linolenate in different known positions. The relative amount of linoleate oxidation was determined by analyzing for hexanal, 2-heptenal and 2,4-decadienal, and the relative amount of linolenate oxidation by analyzing for 2,4-heptadienal and 2,4,7-decatrienal. The volatiles from pure monohydroperoxides of LLL and LnLnLn were compared with those of the corresponding triacylglycerols by capillary gas chromatography. Significant differences in the distribution of volatile products were observed depending on the triacylglycerols precursor. A 1:1 mixture of LLL and LnLnLn autoxidized at 40°C showed an equal contribution of linolenate and linoleate volatiles at a peroxide value of 34. The synthetic triacylglycerols LLnL and LLLn (L, linoleic; Ln, linolenic acid) formed initially about the same total volatiles, whereas LnLnL formed more volatiles than LnLLn. The ratio Ln to L volatile products was the same for the diL triacylglycerols, and higher for LnLnL than for LnLLn. This new information should permit us to better understand the influence of triacylglycerol structure on the relative oxidative stability of unsaturated triacylglycerols.
***Lipids* 27, 442-446 (1992).**

Much work has been reported on the volatile oxidation products of unsaturated fats because they cause rancidity in foods and cellular damage in the body. Decomposition of lipid hydroperoxides creates a wide range of carbonyl compounds, hydrocarbons, furans and other materials that contribute to flavor deterioration of foods (1,2). These materials are also implicated in biological oxidation. Interaction of some of these degradation products with DNA, proteins and enzymes may be involved in cell-damaging reactions (3-5). Although lipid hydroperoxides are the recognized precursors of volatile secondary products, relatively little information is available on the mechanism of their decomposition. Much attention has been given to the effects of processing on oxidative stability of fats and oils, but less attention has been given to the effect of triglyceride structure of polyunsaturated vegetable oils.

In the previous papers of this series, the major autoxidation products of trilinoleoylglycerol (LLL) and trilinolenoylglycerol (LnLnLn) were separated and characterized (6,7). In the preceding paper of this series, four synthetic triacylglycerols containing linoleate and linolenate were autoxidized and the relative amounts of autoxidation products from L and Ln components were investigated by high-performance liquid chromatography (HPLC) techniques (8). This paper describes a study of the effect of triacylglycerol structures on the types and amounts of volatile oxidation products to clarify their contribution to flavor and oxidative deterioration of vegetable oils. Capillary gas chromatography was used as a sensitive method to follow the distribution of volatile oxidation products and to determine the corresponding unsaturated fatty acid precursors.

EXPERIMENTAL PROCEDURES

Materials. LLL and LnLnLn (99+ % purity) were purchased from NuChek Prep, Inc. (Elysian, MN). LLLn, LLnL, LnLnL and LnLLn were synthesized and purified as described previously (9).

Autoxidation. Freshly chromatographed triacylglycerols were autoxidized at 40°C and the pure monohydroperoxides were prepared as described previously (6,7). Peroxide values (PV) were determined by the ferric thiocyanate method (10).

Capillary gas chromatography. The direct injection capillary gas chromatographic (GC) technique was used to analyze volatile decomposition products (11). By this technique samples of oxidized triacylglycerol or monohydroperoxides were injected into a glass insert inside the injector port that was packed with glass wool and heated at 180°C. The volatiles formed were swept into the GC capillary column cooled to −65°C and held for 5 min while the injector was in the splitless mode. The temperature was then programmed to 270°C at 5°C per min, using a fused silica capillary DB-1701 (14% cyanopropylphenyl; 60 m × 0.32 mm) column (J&W, Folsom, CA). The GC conditions were the same as described previously (11).

RESULTS

To investigate the volatiles from triacylglycerols containing linoleic and linolenic acids at different glycerol locations, pure monohydroperoxides of LLL and LnLnLn were decomposed at 180°C under conditions of direct injection GC. Volatile GC analyses of LLL hydroperoxides are compared with those of LnLnLn hydroperoxides in Table 1. Direct injection GC of LLL hydroperoxides produced pentane, hexanal, pentanal, 2-heptenal, 2-octenal and 2,4-decadienal as major volatiles. Direct injection GC of LnLnLn hydroperoxides produced propanal, 2,4-heptadienal and 2,4,7-decatrienal as major volatiles. Other significant volatiles included acrolein, 1-penten-3-ol, 2-pentenal, 2-/3-hexenal and ethyl furanone.

To establish characteristic volatiles of linoleate and linolenate components of triacylglycerols, oxidized LLL, LnLnLn and an equal mixture of LLL and LnLnLn were analyzed quantitatively by direct injection GC at peroxide

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Abbreviations: GC, gas chromatography; HPLC, high-performance liquid chromatography; L, linoleic acid; Ln, linolenic acid; LLL, trilinoleoylglycerol; LnLnLn, trilinolenoylglycerol; PV, peroxide value.

TABLE 1

Capillary Gas Chromatographic Analyses of Volatile Decomposition Products of Hydroperoxides of Trilinoleoylglycerol (LLL) and Trilinolenoylglycerol (LnLnLn)[a]

LLL-OOH			LnLnLn-OOH		
Major volatiles	Area	Rel%	Major volatiles	Area	Rel%
Acrolein	2	0.5	Acrolein	15	5
Pentane	147	27	Propanal	119	36.5
Pentanal	4	1	2-Butenal	6	2
Hexanal	112	20	1-Penten-3-ol	10	3
2-Heptenal	22	4	Ethyl furan	3	1
2-Pentyl furan	3	0.5	2-Pentenal	12	4
2-Octenal	19	3	2-/3-Hexenal	2	0.5
2,4-Decadienal	244	44	Ethyl furanone	15	5
			2,4-Heptadienal	70	21
			2,4,7-Decatrienal	74	22
Total	553	100		326	100

[a]Determined by direct injection capillary GC (see Experimental Procedures). Peak area (× 1000). Average standard deviation of duplicate analyses is 1.0% for LLL-OOH and 1.1% for LnLnLn-OOH. Integrated value for 2-/3-hexenal represents two peaks for each volatile due to the presence of *cis* and *trans* isomers; integrated values for 2,4-decadienal, 2,4-heptadienal, and 2,4,7-decatrienal represent two peaks due to the presence of *cis,trans* and *trans,trans* isomers.

TABLE 2

Major Volatile Decomposition Products of Trilinoleoylglycerol (LLL), Trilinolenoylglycerol (LnLnLn) and of a 1:1 Mixture of LLL and LnLnLn Autoxidized at 40°C[a]

Volatiles Rel%	LLL		LnLnLn		LLL + LnLnLn	
	Area	Rel%	Area	Rel%	Area	Rel%
Pentane	13	45	0	0	8	18
Hexanal	2	7	0	0	1	3
2-Heptenal	3	10	0	0	2	4
2,4-Decadienal	11	38	0	0	12	27
Propanal	0	0	20	47	8	18
2,4-Heptadienal	0	0	10	23	9	19
2,4,7-Decatrienal	0	0	13	30	5	11
Total	29	100	43	100	45	100
Total area/PV	1.2		1.4		1.3	
L Volatiles		100		0		52
Ln Volatiles		0		100		48
Ln/L Volatiles						0.92

[a]Determined by direct injection capillary GC (see Experimental Procedures). Peroxide values for LLL, 22.6; LnLnLn, 31.5; LLL + LnLnLn, 34.3. Peak area (× 1000). Average standard deviation of duplicate analyses is 1.2% for LLL, 2.3% for LnLnLn and 1.3% for LLL + LnLnLn. Integrated values for 2,4-decadienal, 2,4-heptadienal and 2,4,7-decatrienal represent summation of two peaks for each volatile due to the presence of *cis,trans* and *trans,trans* isomers.

values ranging from 22.6 to 34.3. The main volatiles produced from linoleate and linolenate are summarized in Table 2. The main volatiles derived from LLL included pentane, hexanal, 2-heptenal and 2,4-decadienal. The main volatiles derived from LnLnLn included propanal, 2,4-heptadienal and 2,4,7-decatrienal. In the mixture of LLL and LnLnLn, the ratio of linolenate to linoleate volatiles was 0.92 at peroxide value of 34. Therefore, at this peroxide value, the linolenate and linoleate volatiles had about equal contribution to the total decomposition products.

The major volatiles formed from synthetic diL triacylglycerols, LLLn and LLnL, autoxidized at 40°C included in decreasing order: 2,4-decadienal, 2,4-heptadienal, hexanal, 2-heptenal and 2,4,7-decatrienal (Figs. 1a and 1b). The major volatiles formed from synthetic diLn triacylglycerols LnLnL and LnLLn, autoxidized at 40°C included in decreasing order: 2,4-heptadienal, 2,4-decadienal, 2,4,7-decatrienal, hexanal, and 2-heptenal (Figs. 1c and 1d).

The effect of peroxide value on the relative concentrations of these major volatiles is depicted in Figures 2a–e. Among the five volatiles compared, only hexanal showed significant differences between the four triacylglycerols. Hexanal is a major volatile derived from linoleate. Initially, this volatile was formed in larger amounts from LnLLn than LnLnL (Fig. 1a). On the other hand, hexanal was formed in larger amounts from LLLn than from LLnL. Therefore, in the pair of triacylglycerols containing two Ln, more hexanal is formed when L is in the 2 position than when it is in the 1 or 3 position. In the other pair of triacylglycerols containing two L, more hexanal is formed when L is in the 1 and 2 positions than when it is in the 1 and 3 positions.

2,4-Decadienal and 2-heptenal are also volatiles that come from linoleate. No significant differences were found in formation of these two volatiles between the two sets of synthetic triacylglycerols (Figs 2b and 2c). The position of L in these triacylglycerols, therefore, has no effect on the formation of 2,4-decadienal and 2-heptenal. 2,4-Heptadienal and 2,4,7-decatrienal are important volatiles that come from linolenate. No significant differences in the formation of these two volatiles were found between the two sets of synthetic triacylglycerols (Figs. 2d and 2e). The data indicate, therefore, that there is no effect of the position of Ln in these triacylglycerols on the formation of 2,4-heptadienal and 2,4,7-decatrienal.

Figure 3a compares total volatile formation from the synthetic diL triacylglycerols with the corresponding 2:1 mixture of LLL/LnLnLn. About the same amounts of total volatiles were produced by the two synthetic triacylglycerols, and initially both generated less total volatiles than did the 2:1 mixture of LLL/LnLnLn. In contrast, larger differences in total volatiles were observed from the synthetic diLn triacylglycerols and the corresponding 1:2 mixture of LLL/LnLnLn (Fig. 3b). At peroxide values larger than 20, LLnLn produced more total volatiles than the corresponding LnLLn, and both produced more total volatiles than the 2:1 mixture of LLL/LnLnLn. Therefore, the position of Ln in the 1 and 2 position seems to be more important in total volatile formation than that of L in producing total volatiles.

Figure 4 compares the ratios of Ln/L volatiles for the synthetic triacylglycerols. The two diL triacylglycerols show no effect in the relative positions of linoleate in the triacylglycerols. However, the ranges of ratios of Ln/L volatiles for LLLn and LLnL were both higher than the Ln/L ratio in the substrate of 0.5 (Table 3). Therefore, more Ln than L volatiles were produced by LLLn and LLnL than expected from the Ln/L substrate ratio. The Ln/L volatile ratio of the corresponding LLL/LnLnLn mixture of 2:1 was even higher than the Ln/L substrate ratio. On the other hand, the two diLn triacylglycerols show a small difference in the ratio of Ln/L volatiles (Fig. 4). At the beginning and end of the oxidation range, LLnLn pro-

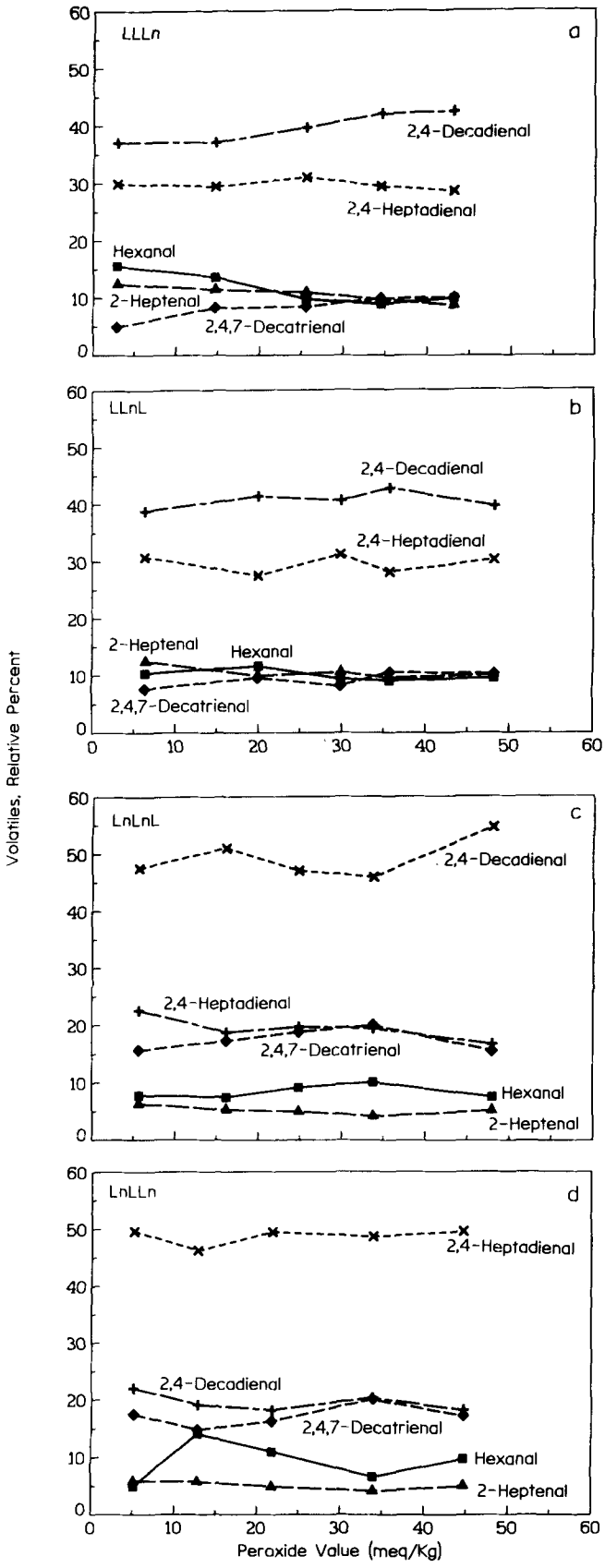

FIG. 1. Autoxidation of synthetic triacylglycerols at 40°C. Analyses by direct injection gas chromatography at 180°C. (a) LLLn; (b) LLnL; (c) LnLnL; (d) LnLLn.

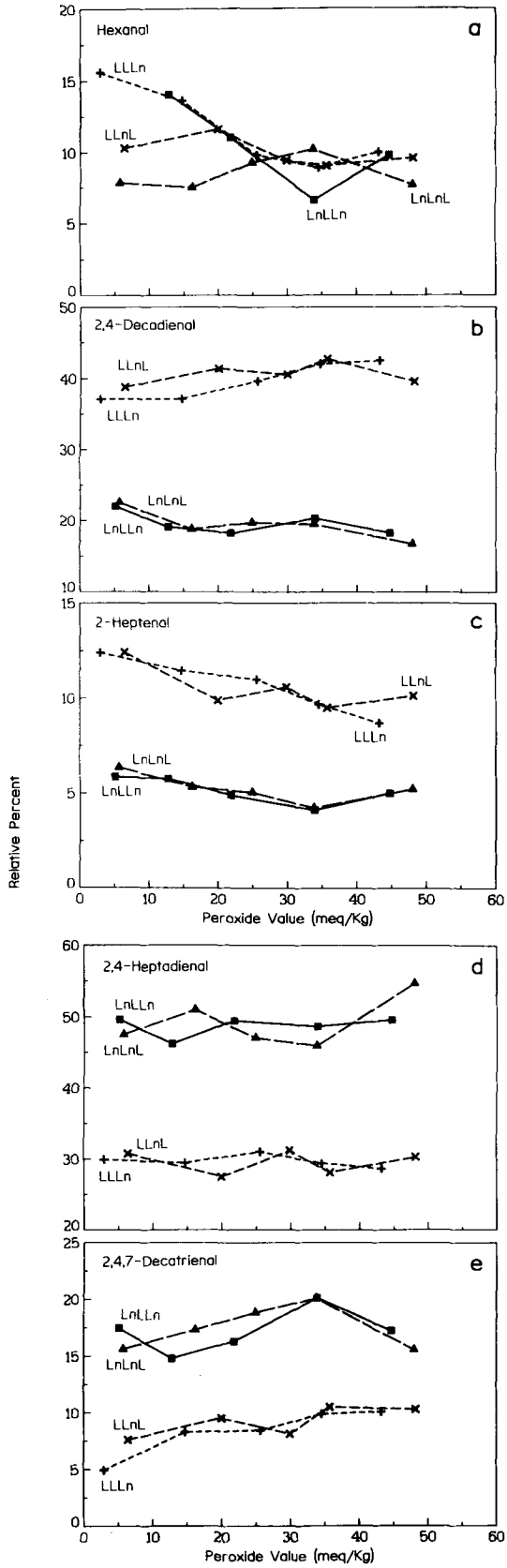

FIG. 2. Autoxidation of synthetic triacylglycerols at 40°C. Analyses by direct injection gas chromatography at 180°C. (a) Hexanal formation; (b)2,4-decadienal formation; (c) 2-heptenal formation; (d) 2,4-heptadienal formation; (e) 2,4,7-decatrienal formation.

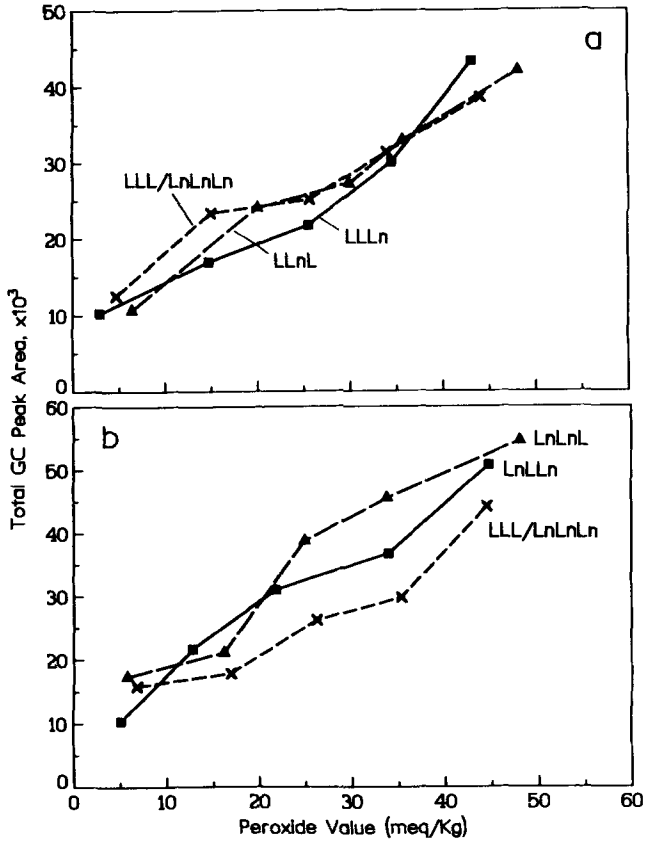

FIG. 3. Autoxidation of synthetic triacylglycerols at 40°C. Analyses by direct injection gas chromatography at 180°C. (a) LLLn, LLnL and a 2:1 mixture of LLL/LnLnLn; (b) LnLnL, LnLLn and a 1:2 mixture of LLL/LnLnLn.

TABLE 3

Relation Between Ratios of Substrates and Volatile Compounds from Synthetic Triacylglycerols and Mixtures of LLL and LnLnLn Oxidized at 40°C[a]

Triacylglycerols	Substrate ratio Ln/L	Volatile compounds Ln/L
LLLn	0.5	0.54-0.65
LLnL	0.5	0.59-0.68
LLL/LnLnLn (2:1)	0.5	0.66-0.84
LnLnL	2.0	1.93-2.37
LnLLn	2.0	1.95-2.21
LLL/LnLnLn (1:2)	2.0	2.37-2.76

[a]Ranges of volatile Ln/L ratios taken from Figure 4.

duced a larger ratio of Ln/L products than the corresponding LnLLn. The ranges of ratios of Ln/L volatiles for LnLnL and LnLLn were about the same as the Ln/L substrate ratio of 2.0 (Table 3). Therefore, the Ln and L volatiles produced by LLLn and LLnL are in the same range as expected from the Ln/L substrate ratio. However, the Ln/L volatile ratio of the corresponding LLL/LnLnLn mixture of 1:2 was significantly higher than the Ln/L substrate ratio. It appears, therefore, that volatile formation from known mixtures of LLL and LnLnLn does not conform to that of the synthetic triacylglycerols. Although

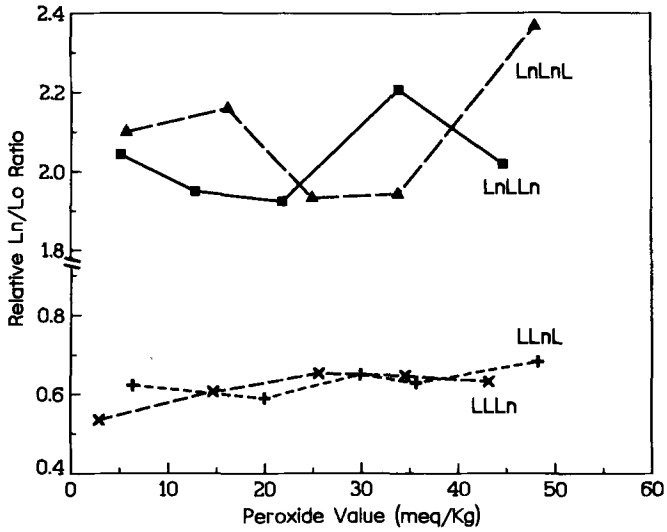

FIG. 4. Autoxidation of synthetic triacylglycerols at 40°C. Analyses by direct injection gas chromatography at 180°C. Relative Ln/L volatile product ratio. Ln volatiles include 2,4-heptadienal and 2,4,7-decatrienal; L volatiles include hexanal, 2,4-decadienal and 2-heptenal.

these mixtures of LLL and LnLnLn have been useful in developing the analytical methodology to distinguish between L and Ln volatiles, they have poor predictive value on volatile formation from triacylglycerols containing L and Ln in different positions.

DISCUSSION

Capillary GC provided a sensitive method to follow the volatile oxidation products of mixtures of LLL and LnLnLn, and of synthetic triacylglycerols containing L and Ln in different known positions. Significant differences in the distribution of volatile products were observed depending on the triacylglycerol precursor. We measured the relative amount of linoleate oxidation by analyzing for hexanal, 2-heptenal and 2,4-decadienal, and the relative amount of linolenate oxidation by analyzing for 2,4-heptadienal and 2,4,7-decatrienal. Mixtures of trilinolein and trilinolenin (1:1) autoxidized at 40°C showed an equal contribution of linolenate and linoleate volatiles at a peroxide value of 34 (Table 2).

The synthetic triacylglycerols LLnL and LLLn initially formed about the same amounts of total volatiles and ratio of linolenate to linoleate products. On the other hand, LnLnL formed more volatiles and a slightly higher ratio of linolenate to linoleate products than LnLnL. In our previous paper (8), when synthetic triacylglycerols were oxidized at 40°C, peroxide formation decreased in the following order: LnLnL > LnLLn > LLnL > LLLn. The oxidation product ratios of linolenate/linoleate showed the following trend: LnLnL > LnLLn >> LLnL = LLLn. We suggested that competitive interactions between two Ln are higher than between Ln and L in adjacent triacylglycerol positions.

In the present work, smaller differences were found in total volatile formation between the synthetic triacylglycerols, and thus smaller effects of the relative positions of L and Ln than in our previous study (8) of peroxide formation using the same set of triacylglycerols. The volatile decomposition products from autoxidized triacylglycerols showed a larger total volatile formation from LnLnL than from LnLLn, but no difference between LLnL and LLLn. The product ratio of Ln/L volatiles showed the same order as that of the peroxide formation (8), *i.e.*, LnLnL > LnLLn >> LLnL = LLLn. Therefore, in the diLn triacylglycerols, two adjacent Ln substituents produced more volatiles than when they are separated by one L substituent. In diL triacylglycerols, the relative acyl position of L had no effect on volatile formation. As for peroxide formation (8), LnLn interactions are apparently also more important for volatile formation than LnL interactions. These interactions are, however, not as significant in the diL triacylglycerols. This new information may permit us to evaluate better the precursors of oxidative deterioration in unsaturated lipids, and to understand the influence of triacylglycerol structure on the relative stability of lipids.

ACKNOWLEDGMENT

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Oxidation of the α-Tocopherol Model Compound 2,2,5,7,8-Pentamethyl-6-chromanol in the Presence of Alcohols

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Oxidation of the vitamin E model compound, 2,2,5,7,8-pentamethyl-6-chromanol (1b) by *t*-butyl hydroperoxide in chloroform has been studied in the presence of ethanol, heptanol and cholesterol. In the absence of an alcohol, the major products were the spirodimer (13b) and spirotrimer (14b) of 1b, together with 1H,2,3-dihydro-3,3,5,6,9,10,11a(R)-heptamethyl-7a(S)-(3-hydroxy-3-methylbutyl)-pyrano[2,3-a] xanthene 8(7aH), 11(11aH) dione (6b). In the presence of ethanol, heptanol and cholesterol, the major products were 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (16b), 5-heptoxymethyl-2,2,7,8-tetramethyl-6-chromanol (17) and 5-cholesteroxymethyl-2,2,7,8-tetramethyl-6-chromanol (18). However, when water was present in a homogeneous reaction, the most rapidly formed product was 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (5b). Compounds 13b, 14b, 16b, 17 and 18 are formed *via* a quinone methide intermediate, and compound 5b is formed *via* a phenoxylium ion. The phenoxylium species appears to be the preferred intermediate when water is present, whereas the quinone methide species is preferred in the absence of water.
Lipids 27, 447–453 (1992).

It has been shown recently that some of the oxidation procuts of α-tocopherol (1a) and of its model compound, 2,2,5,7,8-pentamethyl-6-chromanol (1b), are themselves antioxidants (1). If such compounds are formed *in vivo*, the antioxidant activity of 1a would not necessarily be lost on oxidation, which could help to explain its remarkable efficiency. Oxidation products of 1a and 1b are believed to be formed either through an intermediary phenoxyl radical (2a or 2b; Scheme 1), through a phenoxylium ion (3a or 3b; Scheme 1) or through a quinone methide (8a or 8b, Scheme 1). All of the products with antioxidant activity are derivatives of 8a (or 8b), derivatives of 3a (or 3b) have no antioxidant activity (1). Therefore, the intermediate through which the oxidation proceeds determines whether or not antioxidant activity can be prolonged, and the type of intermediate is determined by the nature of the oxidant and/or of the solvent in which the reaction occurs. Thus the oxidation of 1a and 1b, in an inert, nonpolar solvent, such as petroleum, leads to derivatives of 8a (or 8b) (2,3) such as dimers, trimers and 5-benzoyloxymethyl derivatives (2,4–6). By contrast, oxidation in a protic solvent, such as ethanol, can give derivatives of 3a (or 3b) (7–9), such as quinones (10), 8a-hydroxy-tocopherones (11), 8a-alkoxy-tocopherones (7,12) and quinone epoxides (13), and also derivatives of 8a (or 8b), such as 5-alkoxymethyl compounds (14,15).

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Abbreviations: BzO•, benzoyloxyl radical; BzOH, benzoic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; *t*-BuO•, *t*-butoxyl radical; TLC, thin-layer chromatography.

We have never observed products of 2a,2b, but have found that products of 3a,3b and of 8a,8b are present in all oxidation reactions, and it was the aim of the present work to study the rate of product formation under conditions of varying solvent polarity.

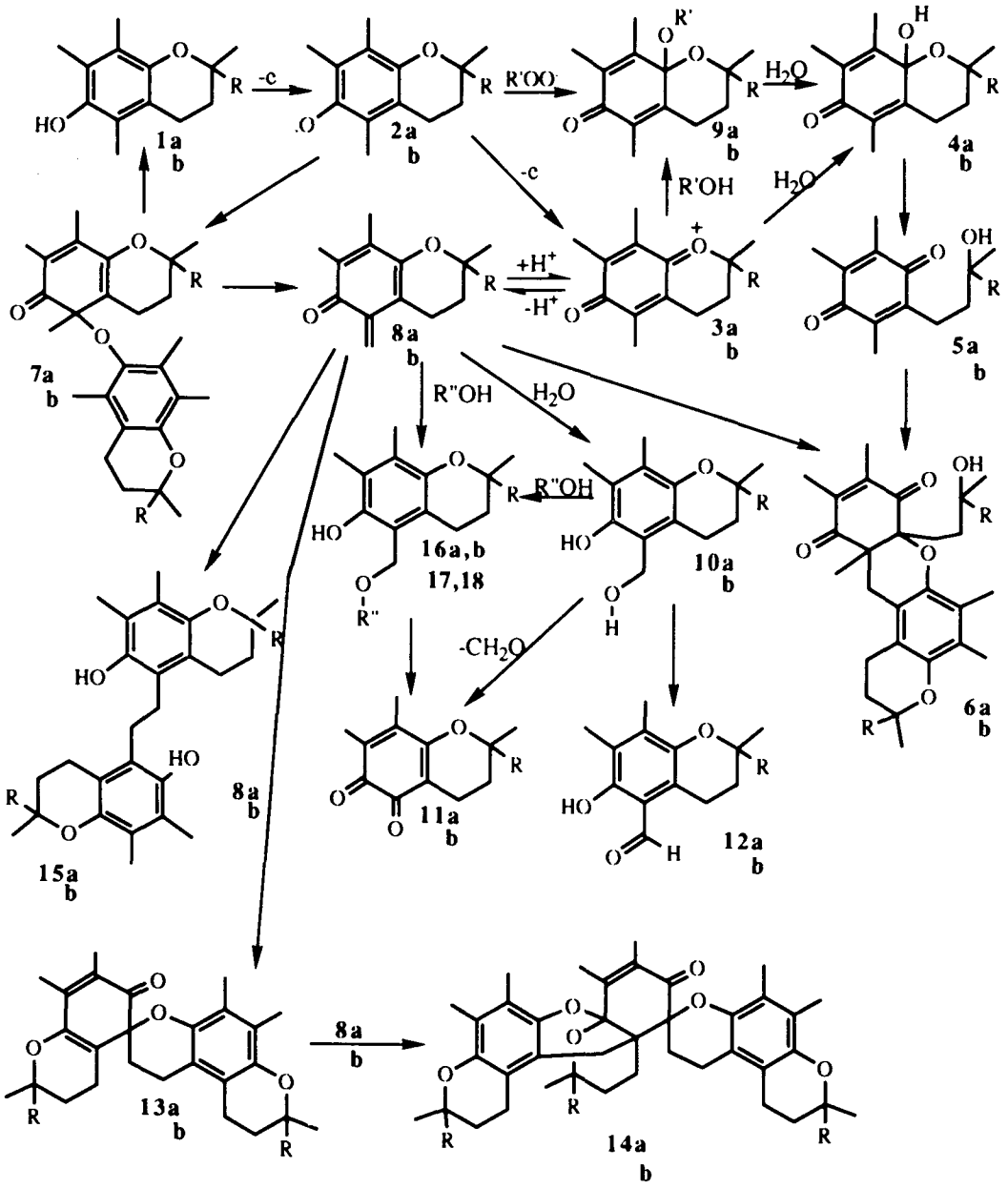
EXPERIMENTAL PROCEDURES

Compound 1b, 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (10b), 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (5b), the spirodimer of 1b (13b), the spirotrimer of 1b (14b), and 1,2-*bis*(2,2,7,8-tetramethyl-6-chromanol-5)ethane (15b) were synthesized to satisfactory purity by established methods (2,5,10,16,17).

The following compounds were isolated from oxidation reactions of 1b: 5-Formyl-2,2-7,8-tetramethyl-6-chromanol (12b) (15); 1H-2,3-dihydro-3,3,5,6,9,10,11a(R)heptamethyl-7a(S)-(3-hydroxy-3-methylbutyl)-pyrano[2,3-a]xanthene-8(7aH), 11(11aH) dione (6b) (18); 5-ethoxymethyl-2,2-7,8-tetramethyl-6-chromanol (16b) (14); 5-heptoxymethyl-2,2,7,8-tetramethyl-6-chromanol (17) (19); 5-cholesteroxymethyl-2,2,7,8-tetramethyl-6-chromanol (18) (19); and 2,2,7,8-tetramethylchroman-5,6-dione (11b) (20).

High-performance liquid chromatography (HPLC) employed a Waters Associates (Milford, MA) 501 (Solvent A) and 6000A (Solvent B) pump, U6K injector, with 2 mL loop, a 660 solvent programer, an ETP Kortec (Sydney, Australia) K 95 variable wavelength detector (set at 290 nm for these experiments), a National Pen Recorder (Matsushita, Yokohama, Japan) VP-6513 A, and a SIC Chromatocorder 12 (Alphatech, Tokyo, Japan) integrator. Separations were performed by linear program (from 7 to 65% Solvent B in 0–40 min) on a Lichrosorb (Merck, Darmstadt, Germany) Si 60, 10 μm column (300 × 4.6 mm; packed in the School of Chemistry, University of New South Wales, Sydney, Australia) at a flow rate of 1 mL/min. The oxidation products had the following retention times (in min) in this system: 18, 3.78; 17, 4.10; 14b, 4.14; 13b, 4.70; 12b, 5.22; 16b, 6.19; 1b, 8.68; 15b, 10.68; 11b, 19.90; 5b, 24.82; 6b, 31.28 (21). Compounds 17 and 14b would have been incompletely separated if they had occurred in the same reaction (*e.g.*, reaction 4 below). However, no trace of 14b or 13b was seen in reaction 4, which had been studied previously by thin-layer chromatography (TLC) (Suarna, C., and Southwell-Keely, P.T., unpublished observations). Solvents A and B were hexane/chloroform (9:1, vol/vol) and hexane/chloroform/ethyl acetate (4:1:5, vol/vol/vol), respectively. Solvents were passed through an Activon (Sydney, Australia) 0.45 μm filter, degassed (Waters Associates pump) and sonicated (Branasonic 12, Branson, Danbury, CT).

Reaction 1. Oxidation of 1b *by t-butyl hydroperoxide in chloroform saturated with water.* A solution of 1b (55.1 mg, 0.25 mmol) in chloroform saturated with water (30 mL) was prepared; an aliquot (2 mL) was taken for analysis as the zero time reaction. *t*-Butyl hydroperoxide (27.0 mg, 0.279 mmol) was added to the remaining solution, and the solution was heated at 60 ± 2°C for 6 h.



a R = $C_{16}H_{33}$
b R = CH_3
R'= alkyl- or alkoxyl-
16 R"= CH_3CH_2-
17 R"= $CH_3(CH_2)_6$-
18 R"= Cholesteryl-

SCHEME 1

Aliquots (2 mL) of the reaction mixture were removed for analysis at 15, 30, 45, 60, 90, 120, 150, 180, 240, 300 and 360 min. Each 2 mL aliquot was washed with 5% ferrous sulfate (3 × 1 mL; to destroy unreacted hydroperoxide) and with distilled water (4 × 1 mL). The aliquots were dried (Na_2 SO_4), and the solvent was removed *in vacuo*. The dried samples were kept over silica gel and under nitrogen at −20°C until ready for analysis. For HPLC, the dried samples were diluted to 2.0 mL with Solvent A and filtered through a 0.45 μm nylon filter (Activon).

Reaction 2. Oxidation of **1b** *by t-butyl hydroperoxide in chloroform containing 20% ethanol.* In a similar manner

to the previous reaction, **1b** (54.9 mg, 0.25 mmol) in a mixture of chloroform (24 mL) and absolute ethanol (6 mL) was oxidized by *t*-butyl hydroperoxide (26.4 mg, 0.27 mmol) at 60 ± 2°C. Aliquots (2 mL) of the reaction mixture were taken at the same intervals for analysis.

Reaction 3. Oxidation of **1b** *by t-butyl hydroperoxide in chloroform containing 18.5% ethanol and 1.5% water.* In a similar manner to the first reaction, **1b** (55 mg, 0.25 mmol), in a mixture of chloroform (24 mL), absolute ethanol (5.55 mL) and water (0.45 mL), was oxidized by *t*-butyl hydroperoxide (27.2 mg, 0.28 mmol) at 60 ± 2°C. Aliquots (2 mL) of the reaction mixture were taken at the same intervals as before for analysis.

Reaction 4. Oxidation of **1b** *by t-butyl hydroperoxide in chloroform containing 20% heptanol.* In a manner similar to the first reaction, **1b** (55 mg, 0.25 mmol), in a mixture of chloroform (24 mL) and heptanol (6 mL), was oxidized by *t*-butyl hydroperoxide (24.1 mg, 0.25 mmol) at 60 ± 2°C. Aliquots (2 mL) of the reaction mixture were taken at the same intervals as before for analysis.

Reaction 5. Oxidation of **1b** *by t-butyl hydroperoxide in chloroform containing cholesterol.* In a manner similar to the first reaction, **1b** (55 mg, 0.25 mmol), in a mixture of chloroform (24 mL) and cholesterol (97 mg, 0.25 mmol), was oxidized by *t*-butyl hydroperoxide (24.0 mg, 0.25 mmol) at 60 ± 2°C. Aliquots (2 mL) of the reaction mixture were taken at the same intervals as before for analysis.

RESULTS

Figure 1 shows that *t*-butyl hydroperoxide oxidizes **1b** very slowly when water is present in a heterogeneous system, recording a 50% loss of **1b** in 100 min and almost complete loss in 360 min. Seventeen products were formed, eight of which have been identified and are shown in Figure 1. The nine additional unknown compounds are not shown. The major product after 360 min was the spirodimer **13b** (40%), and minor products included the pyranoxanthene **6b** (12%), the spirotrimer **14b** (10%), aldehyde **12b** (4.5%), quinone **5b** (3.5%), the 5-ethoxymethyl compound **16b** (2%), the chroman-5,6-dione **11b** (1.5%) and the dihydroxy dimer **15b** (1.6%).

Oxidation of **1b** in the presence of 20% ethanol gives a much faster reaction, with a 50% loss of **1b** in 30 min and almost complete loss in 90 min (Fig. 2). Concomitant with the loss of **1b** is the formation of **16b**, which reaches a maximum of 60% in 90 min and is by far the major product (Fig. 2). The formation of **5b** is unusual in that it forms rapidly (though not as rapidly as **16b**) and reaches a maximum of 4% in the first 30 min (Fig. 2). It then declines, reaching a minimum in 120 min and appears to form again, attaining 3.5% after 360 min. Compounds **11b** and **12b** appear to be secondary products which build up slowly with time reaching 5.7 and 2.7%, respectively, after 360 min (Fig. 2). Three, as yet unidentified, compounds (retention times 4.29, 7.42 and 26.44 min, respectively) also were noted in the reaction (Fig. 2).

Oxidation of **1b** in the presence of 18.5% ethanol and 1.5% water (a homogeneous system) gave an even faster reaction, with a 50% loss of **1b** in 20 min and complete loss in 90 min (Fig. 3). The dominant product early in the reaction was **5b**, which reached a maximum of 50% after 90 min but then fell and was only 17% at 300 min. Compound **16b** was a minor product early in the reaction (8%

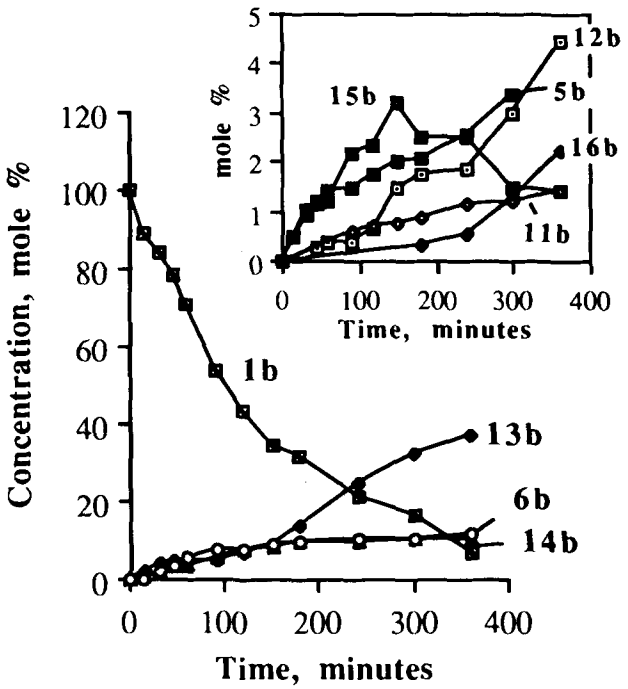


FIG. 1. Formation of oxidation products in the reaction between *t*-butyl hydroperoxide and 2,2,5,7,8-pentamethyl-6-chromanol (1b) in chloroform saturated with water. Products are numbered as in Scheme 1.

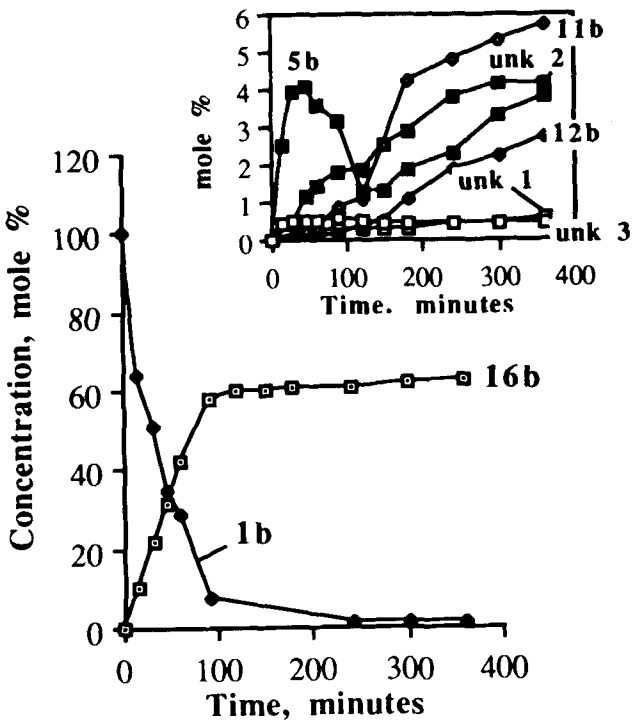


FIG. 2. Formation of oxidation products in the reaction between *t*-butyl hydroperoxide and 2,2,5,7,8-pentamethyl-6-chromanol (1b) in chloroform containing 20% ethanol. Products are numbered as in Scheme 1.

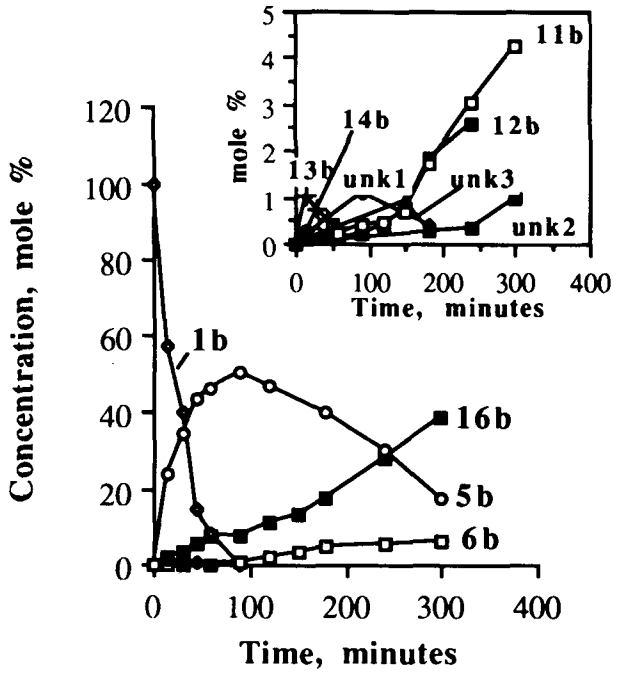


FIG. 3. Formation of oxidation products in the reaction between *t*-butyl hydroperoxide and 2,2,5,7,8-pentamethyl-6-chromanol (1b) in chloroform containing 18.5% ethanol and 1.5% water. Products are numbered as in Scheme 1.

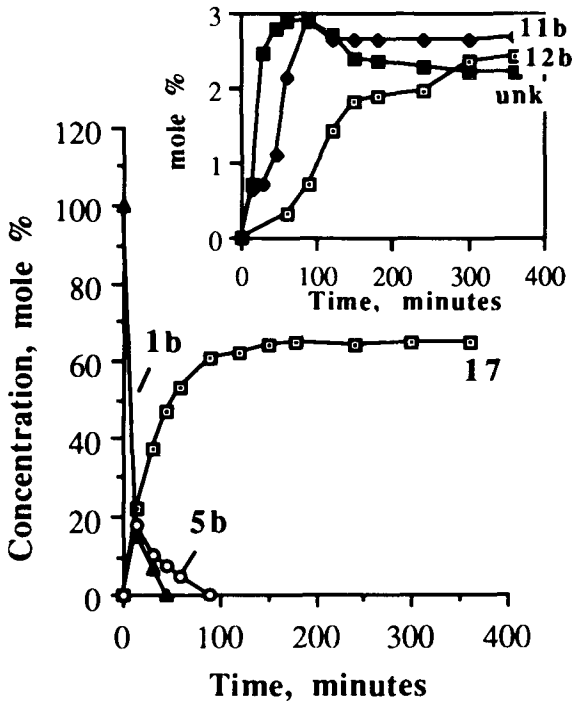


FIG. 5. Formation of oxidation products in the reaction between *t*-butyl hydroperoxide and 2,2,5,7,8-pentamethyl-6-chromanol (1b) in chloroform containing cholesterol. Products are numbered as in Scheme 1.

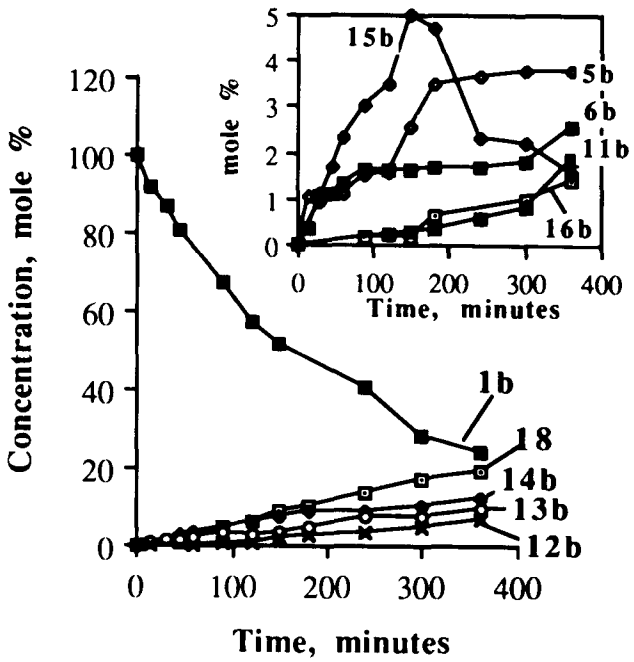


FIG. 4. Formation of oxidation products in the reaction between *t*-butyl hydroperoxide and 2,2,5,7,8-pentamethyl-6-chromanol (1b) in chloroform containing 20% heptanol. Products are numbered as in Scheme 1.

after 90 min) but rose steadily to be the major product (39%) at 300 min (Fig. 3).

Oxidation of **1b** in the presence of 20% heptanol gave the most rapid reaction with 85% loss in 15 min and complete loss in 45 min (Fig. 4). Matching the loss of **1b** was the rapid formation of **17**, which reached 47% in 45 min and a maximum of 64% in 150 min. Compound **5b** also was formed rapidly in the early stages, reaching 18% in 15 min but then dropping to zero in 90 min. Minor products **12b** (2.4% in 300 min) and **11b** (3% in 90 min) were formed again.

Oxidation of **1b** in the presence of cholesterol was a very slow reaction with 50% loss in 150 min and 75% loss in 360 min (Fig. 5). Compound **18** was the major product which was formed at a slow but steady rate to yield 19% after 360 min. As in reaction 1, there was a large number of products of smaller amount ranging from **14b** (12%) to **15b** (1.6%).

DISCUSSION

Oxidation of **1a** and **1b** proceeds by loss of a single electron to form the phenoxyl radicals **2a,2b** (22–24), which may lose a further electron to form the phenoxylium ions **3a,3b** or alternatively dimerize to **7a,7b** followed by disproportionation of the dimers to form one molecule of quinone methide **8a,8b** and one molecule of **1a,1b** (25–27). Since **3a,3b** are the protonated forms of **8a,8b**, respectively, it is likely that an equilibrium exists between the **3** and **8** species. Compounds **2a,2b**, **3a,3b** and **8a,8b** are unstable intermediates and products of each are known. Thus, peroxyl radicals can react with the mesomeric form of **2a** to form **9a** (R′ = O-alkyl) (28–31). Compound **3a** can react with alcohols to form **9a** (R′ = alkyl) (12), or it may react

with water to form **4a**, the precursor of **5a** and **6a** (11). Compounds **8a,8b** are the most versatile intermediates and are the precursors of a wide variety of compounds. They may undergo: i) Diels-Alder polymerization to form **13a, 13b** and **14a,14b** (2,5); ii) Diels-Alder addition to form **6a,6b** (18); iii) nucleophilic addition of water to form **10a,10b** (15), the precursors of **11a,11b** and **12a,12b**; iv) nucleophilic addition of alcohols to form **16a,16b, 17, 18** and other homologs (14,19); or v) disproportionation to form **15a,15b** (5,32).

Previous results have demonstrated that *t*-butyl hydroperoxide is a very weak oxidant for **1b** and will not oxidize it at all unless there is an attendant nucleophile, such as an alcohol or water (14,15). Reaction 1 shows that, even in the presence of a very small amount of water, substantial though slow reaction occurs to form a large number of products.

Products of reaction 1 which are derivatives of the quinone methide **8b** include **11b, 12b, 13b, 14b, 15b** and **16b**. The major derivative of the phenoxylium ion **3b** is **5b**. The pyrano-xanthene **6b** is a derivative of both **8b** and **3b** since it is formed by reaction of **8b** with **5b**. On this basis, derivatives of **8b** account for 64% of **1b** after 360 min, while derivatives of **3b** account for 9.5%. It would seem that the neutral **8b**, much less polar than the ionic **3b**, is the intermediate favored by the nonpolar solvent chloroform. The concentration of dimer **13b** is four times that of the trimer **14b**. By contrast, when **1a** was oxidized by autoxidizing methyl linoleate, the trimer **14a** was the major product and the dimer **13a** the minor product (33). Also, in simpler molecules, the trimer is the preferred product (27). Formation of the trimer probably depends on whether other reactions are available to **8a,8b**. In the present reaction, addition of **8b** to **5b** to form **6b**, and addition of water to **8b** leading ultimately to **11b** and **12b**, compete successfully with trimerization.

Alkaline ferricyanide is the reagent which has been used most in the preparation of spirodimers **13a,13b** (4,5), and a comparison of its oxidizing power with that of *t*-butyl hydroperoxide is interesting. Even taking into account the more concentrated reaction mixtures used in previous work (4,5), alkaline ferricyanide is a much more effective oxidant than *t*-butyl hydroperoxide, producing approximately the same yield of dimer in 3 min at room temperature as that produced by *t*-butyl hydroperoxide in 360 min at 60°C. If one compares the rate of formation of (**5b** + **6b**) with that of (**11b** + **12b**), it is clear that water would prefer to add to **3b** rather than to **8b**.

Formation of compound **16b** (2% after 360 min) requires explanation. Compound **16b** is formed by the reaction of ethanol with **8b**. However, this reaction contains no ethanol and the chloroform solvent was purified to remove the ethanol stabilizer. This concentration of **16b** can be accounted for by assuming that compound **1b** was contaminated by 0.4% (w/w) ethanol of crystallization. Detection of this concentration of ethanol in the ^{1}H NMR (nuclear magnetic resonance) spectrum of **1b** would have required a 1000-fold peak enhancement which was not performed at the time.

There have been a number of studies involving the oxidation of **1a** with alkylperoxyl radicals (28–31). In each case mixed peroxide products such as **9a** (R′ = O-alkyl) have been reported and, in several cases, isolated (29–31). *t*-Butylperoxyl radical reacts with **1b** to form epoxide peroxides of **9b** (R′ = O-*t*-butyl), which are sufficiently stable that they can be isolated by chromatography on silica gel (34). By contrast, *t*-butoxyl radical produces **13b, 14b** and **15b**, but does not produce compound **9b** (R′ = *t*-butyl) or its epoxides (34), presumably because the bulky *t*-butyl group cannot be accommodated so close to the birdgehead (8a) position (12). We have never observed compound **9b** (R′ = O-*t*-butyl) or its epoxides, either in the present reactions or in our previous reactions involving **1b**. This suggests that the oxidant in our reactions was not the *t*-butylperoxyl radical, but the *t*-butoxyl radical.

After 45 min of reaction 2 (containing 20% ethanol), **16b**, a derivative of **8b**, accounted for 33% of **1b**, while **5b**, a derivative of **3b**, accounted for 4% of **1b**. After 90 min, derivatives of **8b**, (**11b, 12b** and **16b**), accounted for 60%, and derivatives of **3b**, (**5b**) accounted for 1% of **1b**. Thus **8b** was again the overwhelmingly favored intermediate despite the much more polar solvent (20% ethanol) which, one might imagine, would favor **3b**. The rate of formation of **5b** was much more rapid in reaction 2 (4% after 30 min) than in reaction 1 (3% after 300 min), even though there was more water (0.7%) in reaction 1 than in reaction 2 (0.2%). The reason for this must be that in reaction 2, the water was in a homogeneous solution, and therefore more available for reaction, whereas in reaction 1, it was in a heterogeneous solution, on the surface of the chloroform, and therefore less available for reaction.

No trace was found of 8a-ethoxy 2,2,5,7,8-pentamethylchroman-6(8aH)-one (**9b**, R′ = CH_3CH_2). It is doubtful that it was formed at all since very little of its breakdown product, **5b**, was noted in the reaction. Thus, these results differ significantly from those observed by Goodhue and Risley (6,12) who oxidized **1a** with benzoyl peroxide. They found 5-benzoyloxymethyl-γ-tocopherol (a derivative of **8a** with structure analogous to **16a, 16b, 17** and **18**) as major product in nonpolar solvents, such as benzene and hexane. However, they found alkoxy homologs of **9a** (R′ = alkyl) (derivatives of **3a**) as the major products in alcohol solvents. The reasons for the differences between our present results and those of Goodhue and Risley (6,12) may have to do with the substrate or with the oxidant. First, we have used **1b** as substrate, whereas they used **1a**. It is not known whether the phytol side chain of **1a** has an influence on the formation of compound **9a**. However, compound **9a** (R′ = alkyl) is well known (12), whereas compounds **9b** (R′ = alkyl) does not appear to have been reported. Second, our oxidant was probably the *t*-butoxyl radical, whereas Goodhue and Risley used the benzoyloxyl radical (BzO•, derived from benzoyl peroxide). When BzO• reacts with **1a**, it forms benzoic acid (BzOH) and **2a** (Eq. [1]):

$$\text{BzO}\bullet + \mathbf{1a} \rightarrow \text{BzOH} + \mathbf{2a} \qquad [1]$$

Compound **2a** disproportionates to form **8a**, as described above. In nonpolar solvents such as benzene and hexane, BzOH acts as a nucleophile and adds to **8a** to form 5-benzoyloxymethyl-γ-tocopherol. However, in protic solvents such as alcohols, it is possible that an equilibrium exists between **3** and **8** (Scheme 1), both of which are capable of reacting with ethanol. Results of reaction 2 suggest that ethanol prefers to react with **8** rather than with **3**. However, if a stronger acid such as BzOH is present,

it may push the **8a** ↔ **3a** equilibrium towards **3a** and thus favor the formation of **9a**.

In our system, the oxidant is probably the *t*-butoxyl radical (*t*-BuO•) which would react with **1b** to form *t*-butanol and **2b** (Eq. [2]):

$$t\text{-BuO}\bullet + \mathbf{1b} \rightarrow t\text{-BuOH} + \mathbf{2b} \qquad [2]$$

Compound **2b** disproportionates to form **8b** as before. *t*-Butanol can act as a nucleophile and add to **8b** to form 5-*t*-butoxymethyl-2,2,7,8-tetramethyl-6-chromanol (analogous to **16b**, **17** and **18**) (19). However, it does not do so in this case, probably because of the overwhelming, competing excess of ethanol which reacts with **8b** to form **16b**. It would appear that neither *t*-butanol nor ethanol is a sufficiently strong acid to drive the **8b** ↔ **3b** equilibrium towards **3b** and, hence, compound **9b** (R′ = CH_3-CH_2) does not form.

There seems to be no relationship between the strength of the oxidant and the type of derivative formed. Thus benzoyl peroxide, which is a stronger oxidant than *t*-butyl hydroperoxide (35), and ferric chloride, which is weaker than *t*-butyl hydroperoxide or benzoyl peroxide, both give rise to **9a**, whereas *t*-butyl hydroperoxide gives rise to **16b**–**18**. Reaction temperature does not appear to be a factor since the benzoyl peroxide reactions were run at 60°C or higher and the present reactions were run at 60°C, whereas the ferric chloride reactions were run at −10°C (7).

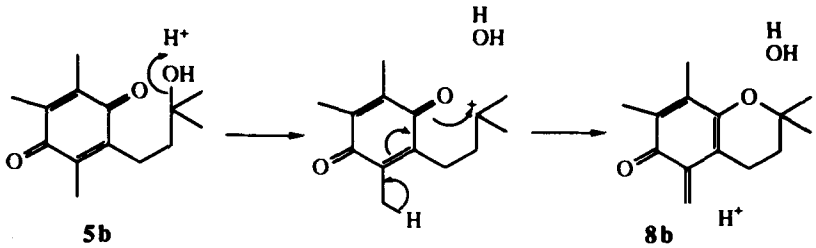
In reaction 3 (containing 18.5% ethanol and 1.5% water), **5b** is formed most rapidly and dominates the early phase of this reaction even though the molar concentration of water is one-fifth that of ethanol, whereas products of **8b** (**11b**, **12b** and **16b**) are formed much more slowly and dominate the later stages. Clearly, both **3b** and **8b** are present at the same time but the increase in water from 0.2% in reaction 2 to 1.8% in reaction 3 heavily favors **3b**. It is possible that water is able to shift the **8b** ↔ **3b** equilibrium towards **3b** because of the much greater ability of water to solvate the ionic **3b** rather than the neutral **8b**.

The results of reactions 1–3 and others (28–31,34) suggest that the first-formed products **2a**,**2b** can react with peroxyl radicals, if present, to form **9a**,**9b** (R′ = O-alkyl). If no species are present which can react directly with **2a**,**2b** they will either lose an electron to form **3a**,**3b** or disproportionate to form **8a**,**8b**. Protic solvents will promote the formation of the **8** ↔ **3** equilibrium. The high yield of **16b** and absence of **9b** (R′ = CH_3CH_2) in reaction 2 suggests that **3** is formed in the sequence **2** → **7** → **8** ↔ **3** rather than the more direct **2** → **3**. If **3** were formed in the sequence **2** → **3**, one would expect **9b** rather than **16b** to be the major product in reaction 2.

In reaction 3, one might ask how **16b** can continue to be formed after **1b** is completely destroyed, since **1b** is the source of **8b**, which reacts with ethanol to form **16b**. Since the concentration of **5b** falls as **16b** rises, one might suspect that **5b** is being converted into **16b**. Compound **5b** is completely stable when heated with *t*-butyl hydroperoxide at 60°C in chloroform. However, **5b** is easily converted into **16b** when heated at 60°C in ethanol in the absence of *t*-butyl hydroperoxide but in the presence of acid (Kohar, I., and Southwell-Keely, P.T., unpublished observations). In order for **5b** to be converted into **16b**, it must proceed through **8b** (Scheme 2). The source of acidity for the **5b** ↔ **8b** conversion in reaction 3 is probably the formation of **5b** itself. Thus, when **3b** reacts with water to form **4b**, the precursor of **5b**, a proton is liberated. Another possible source of **16b** is 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**10b**), which is formed by reaction of water with **8b**. Compound **10b** can react with ethanol to form **16b** (Kohar, I., and Southwell-Keely, P.T., unpublished observations). For reasons which are not completely understood, but which are related to its lability, particularly to acid, **10b** is very difficult to detect by HPLC. However, **10b** must be present since **11b** and **12b** are formed from it. Minor secondary products included **6b** (6% after 300 min), **11b** (4%) and **12b** (3%), each of which began to increase as **5b** began to decline. Each is probably formed as a consequence of the **5b** ↔ **8b** interconversion described above. Again, no trace of **9b** (R′ = CH_3-CH_2) was observed in this reaction. However, it is possible that **9b** may have been formed and converted into **5b**.

Reaction 4, which contained heptanol as the nucleophile, gave the most rapid reaction, the highest yield of any product (**17**, 65%) and the simplest mixture of products, despite the fact that the concentration of heptanol in reaction 4 was only one-third, on a molar basis, that of ethanol in reaction 2. It would appear that the more nonpolar the attacking nucleophile, the more rapid the rate of reaction is likely to be. In the first 15 min, production of **5b** was almost as fast as that of **17**, but it then decreased rapidly while production of **17** continued to increase. Again, as in reaction 2, no trace of **9b** (R′ = CH_3-$(CH_2)_6$) was found. The continuing formation of **17** after **1b** had been completely destroyed may have been due to conversion of **5b** into **8b** under the influence of acid (as in reaction 3), followed by reaction of **8b** with heptanol.

Reaction 5, with cholesterol as the attacking nucleophile, was the slowest reaction of all. However, this would be expected from the much lower molar ratio of cholesterol



SCHEME 2

to **1b** (1:1) as compared with ethanol to **1b** (430:1) and heptanol to **1b** (170:1). Due to its lack of solubility in chloroform, it was necessary to use a much lower concentration of cholesterol than those of the other alcohols. In addition, one might expect a reduction in reaction rate from a large, bulky secondary alcohol (cholesterol) as compared with smaller, less bulky primary alcohols. Nevertheless, the major product was **18** and derivatives of **8b** dominated the reaction. Taken together, the concentration of **13b** and **14b** just exceeded that of **18**, showing that when the nucleophile is large and bulky, polymerization of **8b** competes well with nucleophilic addition, whereas nucleophilic addition predominates with a smaller, more reactive and/or more concentrated nucleophile.

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Triester Glycerothiophosphates of Cholecalciferol (Vitamin D_3)

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***Tris*(*N,N*-dimethyl)amide of phosphorous acid activated by addition of iodine at an optimal molar ratio of 1.05:0.05 was used as a phosphorylating reagent to synthesize cholecalciferyl-3-*O*-(1-2-isopropylidene-*rac*-glycero-3-*O*-)-thiophosphate and cholecalciferyl-3-*O*-(1,2-dipalmitoyl-*rac*-glycero-3-*O*-)thiophosphate of methyl alcohol, 2-dimethylaminoethanol, 3-dimethylamino-1-propanol and 1,2-isopropylidene-*rac*-glycerol in a "one-pot procedure" in overall yields of 60–80%. Activation of the reaction with an equimolar mixture of imidazole and carbon disulfide at the triester formation step permits selective phosphorylation at room temperature. The compounds synthesized represent new triester phospholipid model compounds in which (in addition to glycerol and another requisite alcohol) a steroid and an element other than oxygen are bond to phosphorus.**

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Synthetic model phospholipids containing phosphate triester linkages are of considerable interest to elucidate their chemical, biochemical and pharmacological properties. The synthesis of a triester phospholipid that includes (in addition to glycerol and another alcoholic moiety) a biological active lipid has not been reported as of yet.

In the present paper we describe the synthesis of asymmetrical triester glycerothiophosphates containing vitamin D_3 (Fig. 1). These types of compounds represent new model structures in which a biologically important element other than oxygen also is bonded to phosphorus.

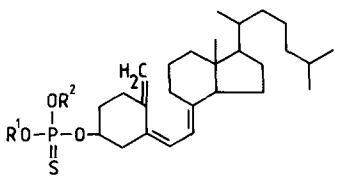

FIG. 1. Triester thiophosphates of vitamin D_3: R^1 = 1,2-isopropylidene-*rac*-glyceryl, or 1,2-dipalmitoyl-*rac*-glyceryl; R^2 = methyl, 2-dimethylaminoethyl, 3-dimethylaminopropyl or 1,2-isopropylidene-*rac*-glyceryl.

MATERIALS AND METHODS

Tris(*N,N*-dimethyl)amide of phosphoruous acid **1** was prepared and freshly distilled as described (1). Cholecalciferol (**a**), 1,2-isopropylidene-*rac*-glycerol (**b**), 1,2-dipalmitoyl-*rac*-glycerol (**c**), methyl alcohol (**d**), 2-dimethylaminoethanol (**e**) and 3-dimethylamino-1-propanol (**f**) were obtained commercially (Aldrich, Milwaukee, WI; Merck, Darmstadt, Germany; Sigma, St. Louis, MO) and were better than 98% pure. All other reagents were purchased from Janssen (Stockholm, Sweden) and were better than 98% pure. Benzene (Merck) was dried over sodium and freshly distilled prior to use. Reaction conditions were kept strictly anhydrous.

All processes were monitored by analytical thin-layer chromatography (TLC) on pre-coated aluminum sheets of Silica Gel 60 F_{254} (Merck). High-performance liquid chromatography (HPLC) was done (Gilson 305 System, equipped with a Gilson 131 refractive index detector; Medical Electronics, Middleton, WI) using a Polygosil 60-7 silica gel column (Scandinaviska Genetik AB, Sweden; 250 × 10 mm). Chloroform (System A), chloroform/methanol (90:10, vol/vol; System B), chloroform/methanol (80:20, vol/vol; System C) and n-heptane/ethyl acetate (80:20, vol/vol; System D) were used as mobile phases.

^{13}C Nuclear magnetic resonance (NMR) spectra were recorded on a Varian XL-300 (Palo Alto, CA) spectrometer at 75.43 MHz. ^{13}C Chemical shifts are reported in ppm relative to tetramethylsilane (TMS). ^{31}P NMR spectra were recorded on the same instrument at 121.42 MHz. ^{31}P Chemical shifts are reported in ppm relative to 85% phosphoric acid (external), where a positive sign is downfield from the standard. Infrared (IR) spectra were recorded on a Perkin-Elmer FT-IR 1750 (Beaconsfield, England) spectrometer. Peak positions are reported in cm^{-1}. Satisfactory microanalyses were obtained for **1abdS, 1acdS, 1aceS, 1abfS** and **1ab²S**: C, ±0.25; H, ±0.10; P, ±0.10; S, ±0.11.

*Cholecalciferyl-3-O-(1,2-isopropylidene-*rac*-glycero-3-O-)-(methyl-O-)thiophosphate,* **1abdS**: *Representative procedure.* A mixture of iodine (0.025 g, 0.1 mmol) and the *tris*(*N,N*-dimethyl)amide of phosphorous acid (**1**; 0.343 g, 2.1 mmol) in benzene (50 mL) was heated at 70°C in a stream of argon for approximately 15 min until the precipitate dissolved. Cholecalciferol (**a**; 0.769 g, 2.0 mmol) was added, and the mixture was kept under these conditions for 10 min to give **1a**. 1,2-Isopropylidene-*rac*-glycerol (**b**; 0.264 g, 2.0 mmol) was then added and the reaction system was heated at 70°C for 1.5 h to give **1ab**. The solution was cooled to room temperature (20–25°C) and added to a mixture of methyl alcohol (**d**; 0.064 g, 2.0 mmol), imidazole (0.136 g, 2.0 mmol) and carbon disulfide (0.152 g, 2.0 mmol) in benzene (50 mL). After 2 h at 20–25°C, the resultant triester phosphite **1abd** was transformed to the thiophosphate **1abdS** by adding sulfur (0.067 g, 2.1 mmol) at 20–25°C for 30 min. The solvent was removed under vacuum, and the compound was isolated by HPLC (System D) in pure form. Yield of **1abdS**: 0.98 g (80%); $n_D^{40} = 1.5098$; R_f (System A), 0.55; $C_{34}H_{57}O_5PS$ (609.0). ^{13}C NMR-{H} ($CDCl_3$) δ 11.9 ppm (C-18); 22.5 (C-26); 22.8 (C-27); 73.9 (*d*, C-3, $J = 9$ Hz); 112.9 (C-19); 117.4 (C-7); 122.9 (C-6); 133.7 (C-8); 142.6 (C-5); 144.1 (C-10): *cholecalciferyl-3-O-fragment;* 25.2, 26.7 (CH_3C); 66.2 (CH_2CHCH_2OP); 67.6 (*m*, CH_2CHCH_2OP); 77.2 (*m*, CH_2CHCH_2OP); 109.7 (CH_3C): *1,2-isopropylidene-*rac*-glycero-3-O-fragment;* 54.4 (*m*, CH_3OP). ^{31}P NMR-{H} ($CDCl_3$) δ 69.2 ppm (m). IR (KBr, film) ν 3030 (CH=);

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Abbreviations: HPLC, high-performance liquid chromatograpy; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane.

1640 (C=C); 1360, 1370 (*gem*-CH_3); 1010, 830 (PO-C, P-OC); 710 cm^{-1} (P=S).

Cholecalciferyl-3-O-(1,2-dipalmitoyl-rac-glycero-3-O-)(methyl-O-)thiophosphate, **lacdS**. The derivative was synthesized using cholecalciferol (**a**; 0.769 g, 2.0 mmol), 1,2-dipalmitoyl-*rac*-glycerol (**c**; 1.138 g, 2.0 mmol) and methyl alcohol (**d**; 0.064 g, 2.0 mmol), and then purified in the same way as described for **labdS** (variant I).

A mixture of the 1,2-isopropylidene-*rac*-glycero-3-*O*-thiophosphate derivative (**labdS**; 1.218 g, 2.0 mmol), palmitoyl chloride (1.100 g, 4.0 mmol), and zinc chloride (0.050 g) was kept at 20–25°C for 24 h. The resulting product was dissolved in diethyl ether (25 mL). The solution was placed on a diethyl ether-filled column of aluminium oxide (30 g; Brockmann II; basic) and the column was washed with diethyl ether (300 mL). The solvent was distilled off and the compound **lacdS** was isolated by HPLC (System D) in pure form (variant II). Yield of **lacdS**: 1.53 g (73%); $n_D^{40} = 1.4761$; R_f (System A), 0.67 (variant I); 0.79 g (38%); $n_D^{40} = 1.4766$; R_f (System A), 0.67 (variant II); $C_{63}H_{113}O_7PS$ (1045.8). ^{13}C NMR-{H} ($CDCl_3$) δ 12.1 ppm (C-18); 22.6 (C-26); 22.8 (C-27); 71.9 (*d*, C-3, $J = 9$ Hz); 112.9 (C-19); 117.6 (C-7); 123.2 (C-6); 133.1 (C-8); 142.3 (C-5); 144.2 (C-10); *cholecalciferyl-3-O-fragment;* 61.8 (CH_2CHCH_2OP); 65.5 (*m*, CH_2CHCH_2OP); 76.5 (*m*, CH_2CHCH_2OP): *glycero-3-O-fragment;* 14.1 (C-16); 173.0, 173.3 (C-1): *palmitoyl-fragment;* 54.5 (m, CH_3OP). ^{31}P NMR-{H} ($CDCl_3$) δ 69.6 ppm (*m*). IR (KBr, film) ν 3030 (CH=); 1740 (C=O); 1640 (C=C); 1010, 830 (PO-C, P-OC); 720 cm^{-1} (P=S).

Cholecalciferyl-3-O-(1,2-dipalmitoyl-rac-glycero-3-O-)(2-dimethylaminoethyl-1-O-)thiophosphate, **laceS**. The compound was prepared using cholecalciferol (**a**; 0.769 g, 2.0 mmol), 1,2-dipalmitoyl-*rac*-glycerol (**c**; 1.138 g, 2.0 mmol) and 2-dimethylaminoethanol (**e**; 0.178 g, 2.0 mmol) following the procedures described for **labdS**. The triester formation stage took 4 h. The crude derivative was purified by HPLC (System B). Yield of **laceS**: 1.69 g (76%); $n_D^{40} = 1.4898$; R_f (System B) 0.68; $C_{66}H_{120}NO_7PS$ (1102.9). ^{13}C NMR-{H} ($CDCl_3$) δ 12.0 ppm (C-18); 22.6 (C-26); 22.8 (C-27); 72.8 (*d*, C-3, $J = 9$ Hz); 112.9 (C-19); 117.6 (C-7); 122.9 (C-6); 133.8 (C-8); 142.3 (C-5); 144.2 (C-10): *cholecalciferyl-3-O-fragment.* 61.8 (CH_2CHCH_2OP); 65.6 (*m*, CH_2CHCH_2OP): *glycero-3-O-fragment;* 14.1 (C-16); 172.5, 172.9 (C-1): *palmitoyl-fragment;* 45.4 (CH_3N); 58.5 (CH_2N); 65.8 (*m*, CH_2OP): *aminoethyl-1-O-fragment.* ^{31}P NMR-{H} ($CDCl_3$) δ 67.5 ppm (m). IR (KBr, film) ν 3030 (CH=); 1746 (C=O); 1647 (C=C); 1005, 831 (PO-C, P-OC); 720 cm^{1-} (P=S).

Cholecalciferyl-3-O-(1,2-isopropylidene-rac-glycero-3-O-)(3-dimethylaminopropyl-1-O-)thiophosphate, **labfS**. The compound was synthesized using cholecalciferol (**a**; 0.769 g, 2.0 mmol), 1,2-isopropylidene-*rac*-glycerol (**b**; 0.264 g, 2.0 mmol) and 3-dimethylamino-1-propanol (**f**; 0.206 g, 2.0 mmol) in the same way as described for **laceS**. The derivative was isolated by HPLC (System C) in pure form. Yield of **labfS**: 0.82 g (60%); $n_D^{40} = 1.5097$; R_f (System C), 0.14; $C_{38}H_{66}NO_5PS$ (680.1). ^{13}C NMR-{H} ($CDCl_3$) δ 12.0 ppm (C-18); 22.5 (C-26); 22.8 (C-27); 73.9 (*d*, C-3, $J = 9$ Hz); 113.0 (C-19); 117.4 (C-7); 122.8 (C-6); 133.6 (C-8); 142.9 (C-5); 143.9 (C-10): *cholecalciferyl-3-O-fragment;* 25.2, 26.8 (CH_3C); 66.0 (CH_2CHCH_2OP); 68.0 (*m*, CH_2CHCH_2OP); 74.8 (m, CH_2CHCH_2OP); 109.3 (CH_3C): *1,2-isopropylidene-rac-glycero-3-O-fragment;* 29.1 (*m*, $OCH_2CH_2CH_2N$); 45.9 (CH_3N); 55.1 (CH_2N); 66.8 (m, 10.2 CH_2OP): *aminopropyl-1-O-fragment.* ^{31}P NMR-{H} ($CDCl_3$) δ 67.7 ppm (*m*). IR (KBr, film) ν 3030 (CH=); 1646 (C=C); 1360, 1370 (*gem*-CH_3); 1005, 835 (PO-C, P-OC); 711 cm^{-1} (P=S).

Cholecalciferyl-3-O-bis(1,2-isopropylidene-rac-glycero-3-O-)thiophosphate, **lab^{2}S**. The intermediate **lab** was prepared using cholecalciferol (**a**; 0.769 g, 2.0 mmol) and then 1,2-isopropylidene-*rac*-glycerol (**b**; 0.264 g, 2.0 mmol) as described for **labdS**. Treatment of a solution of 1,2-isopropylidene-*rac*-glycerol (**b**; 0.264 g, 2.0 mmol), imidazole (0.136 g, 2.0 mmol) and carbon disulfide (0.152 g, 2.0 mmol) in benzene (50 mL) with the intermediate **lab** at 20–25°C for 4 h afforded the triester phosphite **lab^2**. Transformation to thiophosphate **lab^{2}S** was accomplished by reaction with sulfur (0.067 g, 2.1 mmol) at room temperature for 30 min (variant I).

The intermediate **1a** was prepared using cholecalciferol (**a**; 0.769 g, 2.0 mmol) as described for **labdS**. The cooled solution (20–25°C) was then added to a mixture of 1,2-isopropylidene-*rac*-glycerol (**b**; 0.529 g, 4.0 mmol), imidazole (0.272 g, 4.0 mmol) and carbon disulfide (0.304 g, 4.0 mmol) and the reaction system was left at room temperature for 6 h. The transformation to thiophosphate **lab^{2}S** was performed by adding sulfur (0.067 g, 2.1 mmol) at 20–25°C for 30 min (variant II).

The purification procedures were identical with those described for **labdS**. Yield of **lab^{2}S**: 1.08 g (76%); $n_D^{40} = 1.5079$; R_f (System D), 0.28 (variant I); 1.00 g (70%); $n_D^{40} = 1.5085$; R_f (System D), 0.25 (variant II); $C_{39}H_{65}O_7PS$ (709.1). ^{13}C NMR-{H} ($CDCl_3$) δ 12.1 ppm (C-18); 22.7 (C-26); 22.8 (C-27); 73.9 (*d*, C-3, $J = 9$ Hz); 116.1 (C-19); 123.2 (C-7); 125.6 (C-6); 134.6 (C-8); 145.1 (C-5); 150.0 (C-10): *cholecalciferyl-3-O-fragment;* 25.3, 26.7 (CH_3C); 66.3 (CH_2CHCH_2OP); 67.8 (*m*, CH_2CHCH_2OP); 76.4 (*m*, CH_2CHCH_2OP); 109.8 (CH_3C): *1,2-isopropylidene-rac-glycero-3-O-fragment* (Fig. 2). ^{31}P NMR-{H} ($CDCl_3$) δ

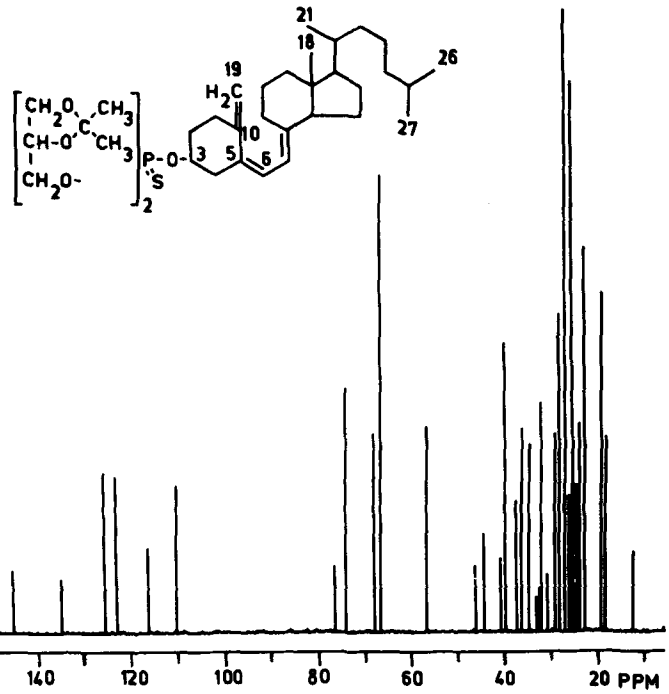


FIG. 2. Proton-decoupled 75.43 MHz ^{13}C NMR spectrum of cholecalciferyl-3-*O*-*bis*(1,2-isopropylidene-*rac*-glycero-3-*O*-)thiophosphate.

68.0 ppm (*m*). IR (KBr, film) ν 3030 (CH=); 1640 (C=C); 1368, 1378 (*gem*-CH_3); 1004, 838 (PO-C, P-OC); 710 cm^{-1} (P=S).

RESULTS AND DISCUSSION

As we have previously shown, changes in the glycerol fragment (1,3-benzylidene-, 1,2-isopropylidene-, 1,3-dioleoyl-, and 1,2-dioleoyl-*rac*-glyceryl), in the position of P–O bonding (glycero-2-O-P-, or glycero-3-O-P-), and in molecular mass had little influence on the stability of the diester glycerothiophosphates of vitamin D_3 (2). In the present report, we examined the possibility of preparing glycerothiophosphates of vitamin D_3 which possess a triester structure. Cholecalciferol **a**, 1,2-isopropylidene-*rac*-glycerol **b**, 1,2-dipalmitoyl-*rac*-glycerol **c**, methyl alcohol **d**, 2-dimethylaminoethanol **e** and 3-dimethylamino-1-propanol **f** were selected as substrates of phosphorylation.

As discussed earlier, the phosphorylation of steroids using conventional reagents is frequently accompanied by a variety of side reactions (3). The acyclic triamides of phosphorous acid, after activation with iodine, afford an opportunity to avoid these problems (3–5). In this case, the *tris*(*N,N*-dimethyl)amide of phosphorous acid **1** with iodine at an optimal molar ratio of 1.05:0.05 was used as reagent.

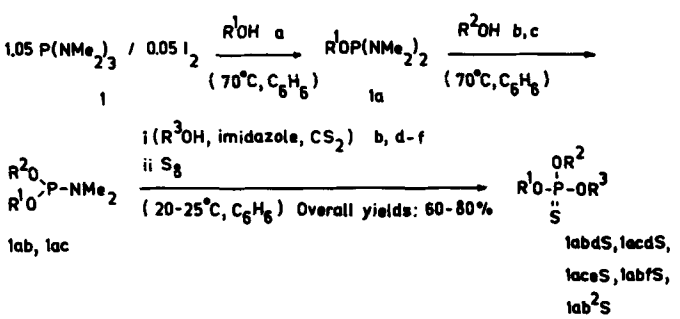
The synthesis was carried out according to the method recently proposed by us (5). The hexamethylphosphorus triamide **1** was reacted with cholecalciferol **a** in stoichiometric amounts at 70°C for 10 min to give the monoester **1a** in close to quantitative yield (Scheme 1). The scheme shows for **a**, **1a**, **1ab**, **1ac**, **1abdS**, **1acdS**, **1aceS**, **1abfS**, and **1ab²S**: R^1 = cholecalciferyl; **b**, **1ab**, **1abdS**, **1abfS**, and **1ab²S**: R^2 (or R^3) = 1,2-isopropylidene-*rac*-glyceryl; **c**, **1ac**, **1acdS**, and **1aceS**: R^2 = 1,2-dipalmitoyl-*rac*-glyceryl; **d**, **1abdS**, and **1acdS**: R^3 = methyl; **e** and **1aceS**: R^3 = 2-dimethylaminoethyl; **f** and **1abfS**: R^3 = 3-dimethylaminopropyl. This was proven earlier by transformation of the phosphite **1a** to the corresponding thiophosphate derivative (2).

The high selectivity of phosphorylation at the monoester formation stage permits the synthesis of asymmetrical diesters in a "one-pot" procedure. The *bis*(*N,N*-dimethylamido)phosphite **1a** was used as the initial key intermediate in order to avoid transesterification under experimental conditions. The crude intermediate **1a** was treated with an equivalent amount of the glycerol derivatives **b** or **c** at 70°C for 1.5 h. In preliminary studies we established that this type of diester phosphite also can be obtained directly in high yields (2). The consecutive treatment of an equimolar mixture of the third substrate **b**, **d**, **e** or **f**, imidazole and carbon disulfide with an equivalent amount of the crude diester intermediate **1ab** or **1ac** and sulfur at room temperature for 2–4 h or 30 min, respectively, afforded the triester thiophosphates **1abdS**, **1acdS**, **1aceS**, **1abfS** and **1ab²S** in good overall yields. The monoester phosphite **1a** also can be reacted directly with two equivalents of the 1,2-isopropylidene-*rac*-glycerol **b** (Scheme 2). In this way we resynthesized compound **1ab²S** (for **1a** and **1ab²S**: R^1 = cholecalciferyl; **b** and **1ab²S**: R^2 = 1,2-isopropylidene-*rac*-glycerol).

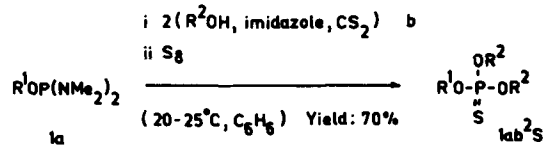
Our attempts to obtain the *bis*(1,2-dipalmitoyl-*rac*-glycero-3-*O*-)thiophosphate and the 1,2-dipalmitoyl-*rac*-glycero-3-*O*-(1,2-isopropylidene-*rac*-glycero-3-*O*-)thiophosphate derivatives of vitamin D_3 failed. The monoester **1a** did not react with two equivalents of 1,2-dipalmitoyl-*rac*-glycerol **c**. The same substrate **c** also was not coupled with the activated diester amidophosphite (type **1ab** or **1ac**) at the last synthetic step. This is probably due to sterical hindrance. The successful synthesis of **1ab²S** would support this hypothesis.

The 1,2-isopropylidene glycerothiophosphate triester can directly be transformed to the corresponding 1,2-diacylglycero-3-*O*- derivatives. For example, treatment of the cholecalciferyl-3-*O*-(1,2-isopropylidene-*rac*-glycero-3-*O*-)-(methyl-*O*-)thiophosphate **1abdS** with two equivalents of palmitoyl chloride in the presence of catalytic amounts of zinc chloride, led to conversion of this triester to its 1,2-dipalmitoyl analogue **1acdS** (Scheme 3; for **1abdS** and **1acdS**: R^1 = cholecalciferyl). However, by this method (6) the target compound was obtained only in low yield (38%).

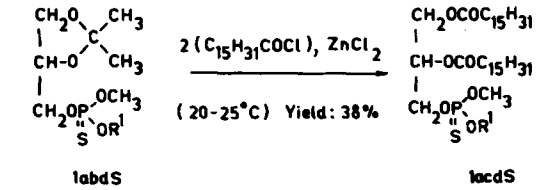
Pronounced downfield shifts were observed for the olefinic carbons of the steroid moiety (C-5 to C-8, C-10, C-19) only in the ^{13}C NMR spectrum of the *bis*(1,2-isopropylidene-*rac*-glycero-3-*O*-)thiophosphate derivative **1ab²S**. In comparison with the other compounds synthesized, C-10 and C-7 were most strongly affected (~5.9 ppm), while the downfield shift was less pronounced for C-19 (~3.2 ppm), C-6, C-5 (~2.7 ppm) and C-8 (~0.9 ppm). It is suggested that the observed spectral features are related to the specific configuration of the phosphorus substituent $(RO)_2P$–, which can induce



SCHEME 1



SCHEME 2



SCHEME 3

marked conformational distortions of the steroid framework. Such long range substituent effects in steroid systems have been discussed extensively (7).

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METHOD

A Colorimetric Assay for Measuring Cell-Free and Cell-Bound Cholesterol Oxidase

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Cholesterol oxidase (cholesterol:oxygen oxidoreductase, EC 1.1.3.6) catalyzes the conversion of sterol Δ^5-3β-alcohol to the corresponding Δ^4-3-ketone with the reduction of oxygen to hydrogen peroxide. *Rhodococcus* species GK 1, a soil isolated microbe, produces an extracellular and a membrane-bound cholesterol oxidase; the latter is bound to the outer surface of the microbial cell membrane. A simple and sensitive assay is described to measure the two enzyme types; no enzyme extraction is needed for measuring the membrane-bound cholesterol oxidase. In this assay, hydrogen peroxide is reduced by the chromogen 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate) (ABTS) in the presence of horseradish peroxidase, and the increased absorbance is followed continuously at 600 nm ($\varepsilon_m = 1.82 \times 10^4$ $M^{-1} \cdot cm^{-1}$ at pH 7.0 and 30°C). The standardized assay medium contained 46.9 mM sodium-potassium phosphate buffer pH 7.0, 0.16% Triton X-100, 312.5 μM ABTS, 50 μg peroxidase (12.5 units at 25°C), 6.25% isopropanol, 306.3 μM cholesterol or other sterols (kept in solution with isopropanol), and cholesterol oxidase. Oxidation of one molecule of cholesterol by cholesterol oxidase gives one molecule of hydrogen peroxide which reacts with two molecules of ABTS. The method is reproducible and the results correlate well with those obtained by measuring the absorbance of Δ^4-cholest-3-one at 240 nm ($\varepsilon_m = 1.40 \times 10^4$ $M^{-1} \cdot cm^{-1}$ at pH 7.0 and 30°C) and by the method of Allain *et al.* (*Clin. Chem. 20*, 470–475, 1974). In terms of efficiency, simplicity, and time saved, this coupled assay is expected to be a useful method for monitoring microbial production of cholesterol oxidase on an industrial scale, and for determining cholesterol or other sterols in biological fluids.

***Lipids 27*, 458–465 (1992).**

3β-Hydroxysteroid oxidase catalyzes the conversion of the Δ^5-3β-hydroxyl group of sterol to the corresponding Δ^4-3-ketone with concomitant reduction of oxygen to hydrogen peroxide. The enzyme is commonly known as cholesterol oxidase (cholesterol:oxygen oxidoreductase, EC 1.1.3.6). Preparations of cholesterol oxidase have been commercially produced from several microbial agents and used for steroid analysis, and particularly for the clinical estimation of plasma cholesterol (1–4).

In many microorganisms, such as *Nocardia* species (4,5) and *Rhodococcus* strains isolated from food of animal origin (butter, bacon, pork fat, and chicken fat) (6), cholesterol oxidase was shown to be an intrinsic membrane-bound type which can be extracted from cells by treatment with nonionic surfactant, such as Triton X-100. In contrast, extracellular cholesterol oxidase was isolated from the broth filtrates of various microorganisms such as *Streptomyces violascens* (7) and *Streptoverticillium cholesterolicum* (8). The microbe *Rhodococcus* species GK1, which we isolated from soil, degraded soybean sterols rapidly during growth and produced very large quantities of membrane-bound cholesterol oxidase. An extracellular form of the enzyme was also detected in the broth filtrate of this strain (Kreit, J., Lefebvre, G., and Germain, P., manuscript in preparation).

The present paper describes an assay for measuring cell-free and cell-bound cholesterol oxidase activities. The procedure is based on the use of 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate) and peroxidase for the reduction of the hydrogen peroxide generated by the cholesterol oxidase reaction. The data show that the method is efficient and simple, and may be useful for monitoring enzyme production on an industrial scale, and for determining sterols in biological fluids.

MATERIALS AND METHODS

Chemicals. Cholesterol, 4-cholesten-3-one, stigmasterol, Carbowax (Polyethylene glycol 6000), phenol and isopropanol were obtained from Fluka AG (Buchs, Switzerland). An alternative commercial source for isopropanol was Carlo Erba (Milan, Italy). β-Sitosterol was purchased from Serva (Heidelberg, Germany). A nonanalytical grade of soybean β-sitosterol (containing other sterols) was supplied by Merck (Darmstadt, Germany), and used only for culturing. 4-Aminoantipyrine (AAP), bovine serum albumin, Lubrol PX and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azino-*bis*(3-ethylbenzothiazoline-6-sulfonate (ABTS), crystallized diammonium salt, and horseradish peroxidase (POD), lyophilized, salt free, 250 units/mg protein at 25°C, were obtained from Boehringer Mannheim (Mannheim, Germany). Hydrogen peroxide, a solution of about 30%, was obtained from Prolabo (Paris, France), and Lubrol WX from Supelco Inc. (Bellefonte, PA). All other chemicals were of analytical grade and were obtained from commercial sources. The buffer solution used was 0.05 M sodium-potassium phosphate (a mixture of Na_2HPO_4 and KH_2PO_4) buffer, pH 7.0–7.1.

Apparatus. Absorbance measurements and spectra were performed by using a Pye Unicam PU 8600 UV/Vis (Pye Unicam Ltd., Cambridge, England), or a Varian DMS 80 UV/Vis (Varian Techtron Pty. Ltd., Melbourne, Australia) spectrophotometer.

Hydrogen peroxide determination. The initial concentration of H_2O_2 in commercial solutions was determined by titration with potassium permanganate ($KMnO_4$) in acidified medium at 0–1°C (9).

Microorganism and cultivation. The strain GK 1, an *ac-*

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Abbreviations: AAP, 4-aminoantipyrine; Abs, absorbance; ABTS, 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate) diammonium salt; CV, coefficient of variation; EU, enzyme unit; POD, peroxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7); UV, ultraviolet.

tinomycete isolated from a polluted soil (Nancy, France) and identified as *Rhodococcus* species, was used. The strain was cultured in a mineral salt medium containing, 3 g $(NH_4)_2SO_4$, 4.16 g Na_2HPO_4, 1.2 g KH_2PO_4, 0.05 g yeast extract, 0.8 mg thiamine per liter, and other mineral ingredients (10). The pH of the medium was adjusted to 7.0 and nonanalytical β-sitosterol (5 g/L) was added as a fine powder directly to the culture flask. Sterilized medium was inoculated and the microbial growth maintained semiaerobically in Erlenmeyer flasks at 30°C with shaking. Microbial growth was stopped in the primary stage of the stationary phase (after about 100 hr of cell growth). Cells were harvested by centrifugation, washed with cooled buffer containing isopropanol (5%) and resuspended in the buffer at 0.5 to 0.8 g wet biomass (the wet weight is about 4 times the dry weight) for 10 mL.

Preparation of cholesterol oxidase solutions. Supernatant from the microbial culture (broth filtrate) was clarified by centrifugation at 56 600 × *g* for 30 min at 5°C, and used as extracellular enzyme source. Cell-bound cholesterol oxidase was extracted by magnetic stirring of the cellular suspension containing 1% Triton X-100 (v/v) for 30 min at room temperature. The enzymic solution was collected by centrifugation and further clarified by recentrifugation at 56 600 × *g* for 30 min.

Concentration of enzyme solutions. When required, cholesterol oxidase solutions were concentrated by protein precipitation with solid ammonium sulfate at 60% saturation. The mixture was stirred for 30 min at 0°C, and then left at this temperature for several hours. The precipitate was collected by centrifugation, dissolved in a small portion of the phosphate buffer and dialyzed overnight against the same buffer at 2–4°C.

Conservation and stability of the enzyme. The cellular suspensions and the enzyme solutions were maintained at 0–2°C. There was no loss in the enzyme activity of these fractions after one month of conservation. The enzyme fractions retained full activity over 2 hr at 30°C.

Standard assays of cholesterol oxidase. The enzyme activity was measured at 30°C and pH 7.0 by estimation of either 4-cholesten-3-one or H_2O_2; both are produced stoichiometrically by the enzyme reaction.

Estimation of 4-cholesten-3-one is based on its ultraviolet (UV) absorption. The enzyme was assayed in a final volume of 3.2 mL containing 150 μmoles sodium-potassium phosphate buffer, 0.16% Triton X-100 (v/v), 0.98 μmole cholesterol (0.2 mL of cholesterol solution in isopropanol), and an appropriate amount of enzymic samples. The reagents were added directly into the spectrophotometer cuvette (1 cm path length). The assay medium was mixed by inversion after adding the substrate and again after adding the enzyme. The cholesterol solution must be deposited onto the surface of the assay medium, and mixing must be rigorous to allow formation of stable detergent-cholesterol micelles giving rise to a clear medium. The rate of increase in absorbance was monitored continuously at 240 nm. The control assay was either without cholesterol or without cholesterol oxidase. Under these conditions, a value of 1.40×10^4 $M^{-1} \cdot cm^{-1}$ was determined for the molar absorption coefficient (ε_m) of 4-cholesten-3-one. Under the same conditions, a value of 38 $M^{-1} \cdot cm^{-1}$ was estimated for ε_m of H_2O_2 at 240 nm. This value is very small and has no effect on cholesterol oxidase evaluation.

H_2O_2 was measured under the conditions mentioned above except that 1 μmole ABTS and 50 μg POD were added to the assay medium (3.2 mL final volume) before cholesterol addition. H_2O_2 produced during the enzyme reaction oxidizes ABTS in a reaction catalyzed by POD. The rate of color formation due to the oxidized ABTS was followed continuously at 600 nm. Commercial H_2O_2 was used under these conditions to determine ε_m of the color; the value obtained was 1.82×10^4 $M^{-1} \cdot cm^{-1}$ with respect to H_2O_2 concentration.

One unit of cholesterol oxidase activity is defined as the amount of enzyme producing 1 μmole of 4-cholesten-3-one (or H_2O_2) per min.

Solution of 2% Triton X-100, 10 μmoles/mL ABTS and 1 mg/mL POD were prepared separately in 0.05 M phosphate buffer pH 7.0 and kept at 2–4°C. These preparations were stable for about 1 year.

Protein determination. Protein was determined by the Biuret method (11). Bovine serum albumin was used as standard. Triton X-100 did not affect the protein determination.

RESULTS AND DISCUSSION

Stadtman and coworkers (12,13) described a microbial dehydrogenase (cholesterol oxidase) for use in a cholesterol assay based on measuring increased absorbance at 240 nm due to formation of 4-cholesten-3-one. Much has been published on this subject, and major efforts have been made to overcome the fact that cholesterol is not water-soluble (14,15). We set out to find conditions for such an assay which would be relatively simple. We studied the effect of isopropanol and of detergents on the solubility of cholesterol in the test medium and on enzyme activity. The essential observations made in this study are summarized here.

Cholesterol oxidase was assayed in the presence of 6 to 35 percent isopropanol, with cholesterol amounts ranging from 6.5 to 260.9 μM, by following the rate of change in absorbance at 240 nm. Only a small amount of substrate (less than 65.2 μM) can be used with 10% isopropanol as higher concentrations result in the formation of cholesterol crystals. Cholesterol was better dissolved in 35% isopropanol; however, the assay was still not adequate.

Secondly, we investigated the effect of the nonionic detergent, Triton X-100, on the cholesterol oxidase assay. The purpose of this was to determine the Triton amounts that would allow formation of stable mixed micelles of detergent and substrate without inhibiting the enzyme. The test mixture contained phosphate buffer, cholesterol oxidase, 6.25% isopropanol, Triton X-100 (0.05–0.35%) and cholesterol (3.8–312.5 μM). Favorable results were obtained with 0.15 to 0.20% Triton X-100 in the assay medium.

The conditions for the standard assay reported in Materials and Methods were derived from the experiments described above. Due to the significant absorption of both Triton X-100 and protein at 240 nm, the assay requires a more sophisticated UV spectrophotometer with good resolution. We therefore tried to replace Triton with either Lubrol WX or Lubrol PX nonionic detergents which do not interfere with UV absorption measurements. Triton X-100, however, was better at keeping cholesterol in solution and maintaining assay linearity. The 4-cholesten-3-

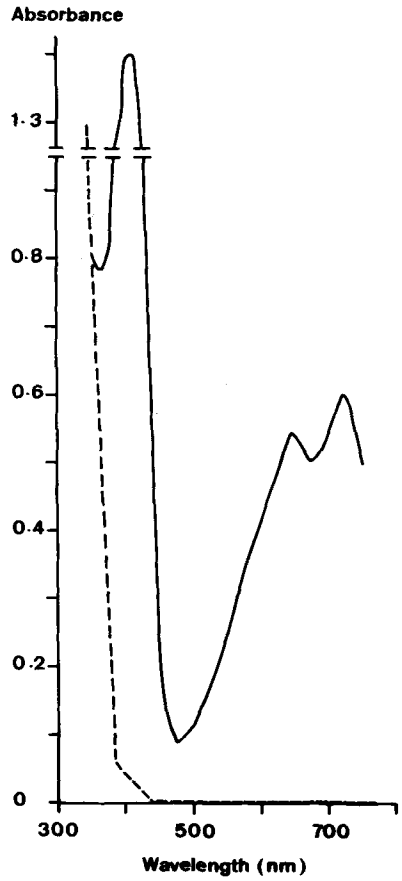

FIG. 1. Visible absorption spectra of ABTS. ABTS (0.136 μmole) was oxidized under the standard assay conditions of the cholesterol oxidase assay in the presence of 14 μmoles H_2O_2 (cholesterol and cholesterol oxidase were absent). After 5 min, when all ABTS was oxidized (the absorbance at 600 nm became constant), a wavelength scan of the cuvette (1 cm path length) was recorded against distilled water (——). For the reduced ABTS spectrum (———), H_2O_2 (or POD) was omitted from the assay medium.

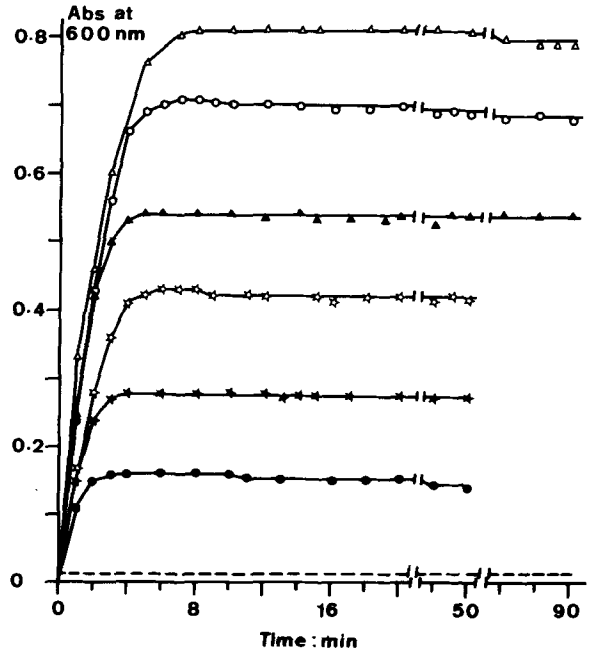

FIG. 2. Stability of final color produced by ABTS oxidation. The standard assay medium contained 0.028 enzyme unit (EU) of cholesterol oxidase (Triton extract, 32 μg protein) and cholesterol. Concentrations of cholesterol (in μmole/assay) were: ●, 0.025; ★, 0.049; ☆, 0.074; ▲, 0.098; ○, 0.123; △, 0.15. The control (———) was without cholesterol. Absorbance was followed against distilled water.

one produced in these studies was identified by gas chromatography (data not shown).

Alternatively, a colorimetric assay method was worked out for measuring cholesterol oxidase activity. In this assay, H_2O_2 produced by the oxidase reaction is used to oxidize the chromogen ABTS in a reaction catalyzed by POD. POD has a high specificity for the electron acceptor H_2O_2. However, it has a low specificity for the electron donor. Several chromogens, such as pyrogallol, *O*-dianisidine, 4-aminophenazine, 3,3′-diaminobenzidine, guaiacol, and ABTS are enzyme active substrates serving as electron donors. In their report on peroxidase steady-state kinetics, Childs and Bardsley (16) stated that most of the electron donors that have been used are much less efficient than ABTS. Since ABTS is also chemically stable and nontoxic, we chose it for the coupled assay of cholesterol oxidase. The optimum amounts of ABTS and POD for the standard assay we determined are those described in Materials and Methods. The validity of the coupled assay for cell-free and cell-bound cholesterol oxidase was ascertained as described below.

Visible absorption spectra of oxidized and reduced

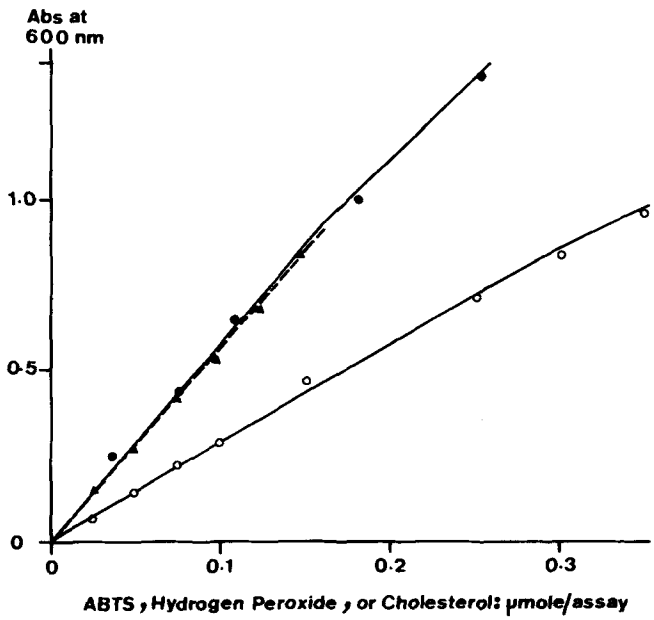

FIG. 3. Plots of oxidized ABTS absorbance for various concentrations of ABTS, H_2O_2, or cholesterol. The standard assay medium (in a cuvette of 1 cm path length) contained 0.361 μmole H_2O_2 for limiting amounts of ABTS (○), or 1 μmole ABTS for limiting concentrations of H_2O_2 (●); cholesterol and cholesterol oxidase were absent. One μmole of ABTS was used for limiting amounts of cholesterol (▲).

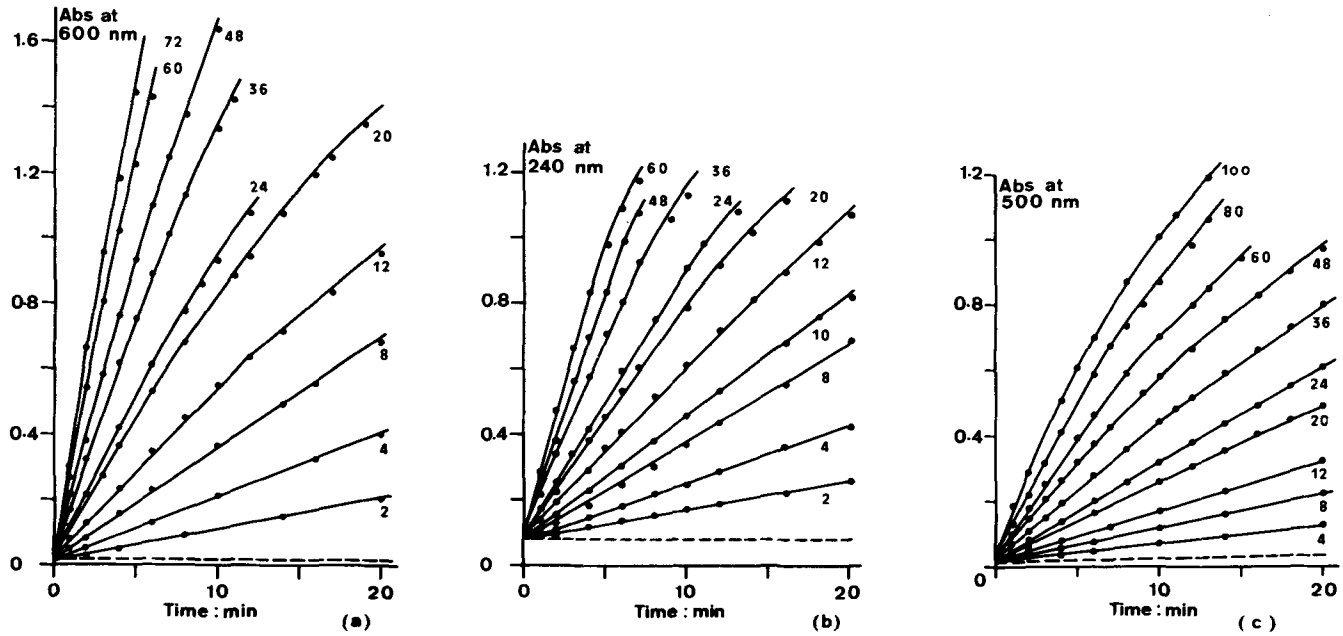

FIG. 4. Measurement of cell-free cholesterol oxidase: comparison studies. Portions from an enzymic Triton extract (1.2 mg protein per mL) were assayed by either the ABTS assay (a), the UV absorption method (b) or by the method of Allain *et al.* (1) (c). The data in panel 4c were obtained using 3.2 mL of final volume containing 280 μmoles phosphate buffer pH 7.0, 44.8 μmoles phenol, 2.53 μmoles AAP, 100 μg POD, 0.16% Triton X-100, 0.98 μmole cholesterol (in 0.2 mL isopropanol) and the enzyme extract. The protein amounts (μg/assay) used are indicated on the plots. Control assays (———) were without cholesterol oxidase. Absorbance was followed against: (a,c) distilled water, (b) the assay medium devoid of cholesterol (or cholesterol together with the enzyme for the control assay).

ABTS were defined with limited amounts of this compound. Figure 1 shows an example of the type of spectra observed. Oxidized ABTS has an absorption maximum at 415 nm ($\varepsilon_m = 3.3 \times 10^4$ $M^{-1} \cdot cm^{-1}$) together with lesser maxima at 640 nm ($\varepsilon_m = 1.25 \times 10^4$ $M^{-1} \cdot cm^{-1}$) and 725 nm ($\varepsilon_m = 1.40 \times 10^4$ $M^{-1} \cdot cm^{-1}$) and a shoulder at 580 nm. Reduced ABTS did not absorb to a significant extent in the range of 400 to 750 nm. The wavelengths at which absorptions are maximal were considered appropriate for measuring the chromogen in the oxidase system. We used a wavelength between 600 and 650 nm for the present purpose ($\varepsilon_m = 0.91 \times 10^4 - 1.24 \times 10^4$ $M^{-1} \cdot cm^{-1}$ with respect to ABTS concentration).

The color stability of oxidized ABTS was examined under the standard conditions of the cholesterol oxidase assay. With limited amounts of cholesterol, 0.025–0.15 μmole per assay, the chromogen absorbance became maximal in 2 to 10 min (Fig. 2) and stayed constant during the time of the test (90 min). When ABTS was the limited factor (0.03 to 0.35 μmole per assay) instead of cholesterol, the final color was stable for 20 min; beyond this time the decline in color absorbance became notable reaching 10% of the original value after an additional 10 min. The same stability of the chromogen color was observed for the limited amounts of ABTS when 0.36 or 14 μmoles H_2O_2 per assay were used instead of cholesterol and cholesterol oxidase. With limited amounts of H_2O_2, 0.03 to 0.2 μmole per assay (cholesterol and cholesterol oxidase were absent), the final color of the chromogen was stable for the entire time of the test (30 min).

The redox capacity of ABTS with H_2O_2 as electron acceptor was investigated under the standard conditions of the cholesterol oxidase assay. Experiments carried out for this purpose were done with limited amounts of either cholesterol or ABTS or H_2O_2; an example is shown in Figure 3. The molar absorption coefficient obtained for oxidized ABTS is 0.91×10^4 $M^{-1} \cdot cm^{-1}$ at 600 nm. A double value was estimated for this coefficient with respect to the concentration of either H_2O_2 or cholesterol instead of ABTS. This result indicated that one molecule of cholesterol (5-cholesten-3β-ol), catalyzed by the oxidase, generated one molecule of H_2O_2 which, in turn, reacted with two molecules of ABTS. Thus, the following reaction scheme can be postulated:

$$\text{5-Cholesten-3}\beta\text{-ol} \xrightarrow{O_2} \text{4-Cholesten-3-one} + H_2O_2$$

$$H_2O_2 + 2\ \text{ABTS} \longrightarrow 2\ H_2O + 2\ \text{ABTS}^+$$

The accuracy of the ABTS assay was examined by measuring cell-free cholesterol oxidase of *Rhodococcus* species and by comparing the results with those obtained by the UV absorption method (standardized assay) and by the AAP assay of Allain *et al.* (1). According to the last procedure, H_2O_2 generated by the enzymic oxidation of cholesterol is measured by the oxidative coupling of AAP with phenol catalyzed by POD to yield a quinoneimine dye with a maximum absorption at 500 nm ($\varepsilon_m = 5.33 \times 10^4$ $M^{-1} \cdot cm^{-1}$). The method of Allain *et al.* (1) has been widely used for measuring cholesterol oxidase (7,8,17,18); so we chose it for comparison as a currently available assay. However, in this assay Carbowax failed to keep cholesterol in clear suspension at the enzyme-

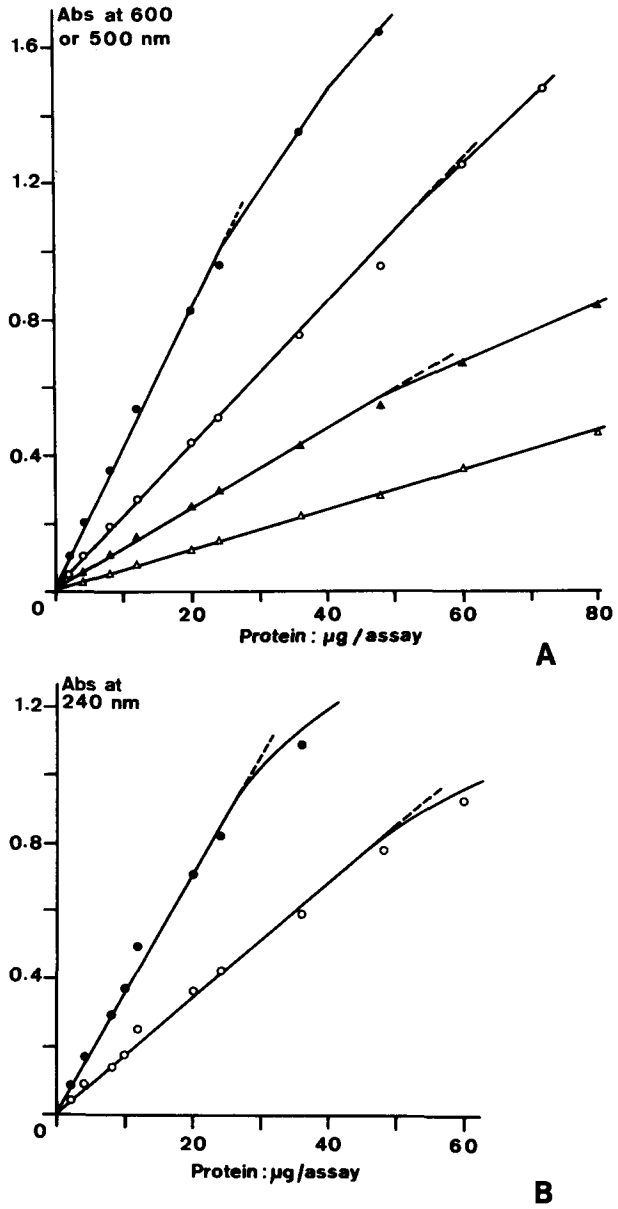

FIG. 5. Cell-free cholesterol oxidase measurement: comparison studies. Data on enzyme activities were derived from Figure 4 for the reaction time of 5 min (empty symbols) and of 10 min (full symbols). a: ○, ●, ABTS assay; △, ▲, AAP assay; b: UV absorption method.

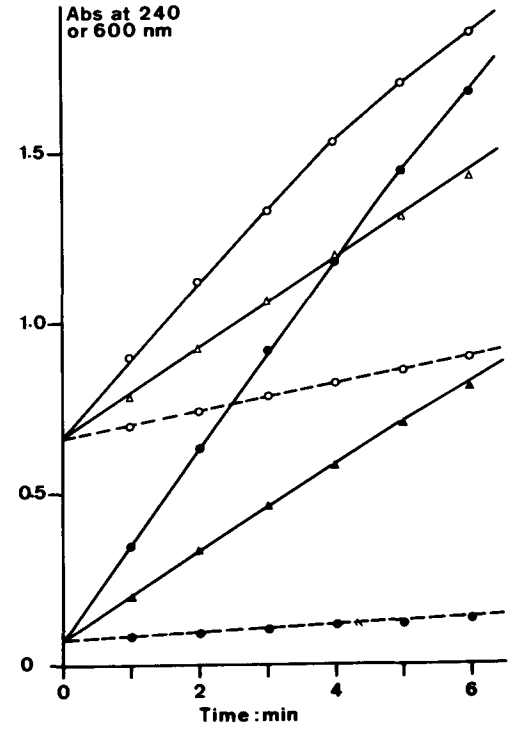

FIG. 6. Activity measurements of cholesterol oxidase with β-sitosterol. The enzyme (37.5 and 75 µg of a Triton extract) was assayed with 0.98 µmole β-sitosterol as substrate. Control assays (dashed lines) were performed without the enzyme. ●, ▲, ABTS assay; the absorbance was followed at 600 nm against distilled water. ○, △, UV absorption method; the absorbance was followed at 240 nm against the test medium devoid of the substrate (or the substrate together with the enzyme for the control assay).

saturating concentrations. Thus, we replaced it with Triton X-100. The results obtained are shown in Figures 4 and 5. The UV absorption method and the ABTS assay were linear up to 0.9 and 1.0 absorbance unit, respectively. This is better than what was observed with the AAP assay (linear up to 0.6 abs. units). The control of the ABTS assay with cholesterol as substrate showed a very low absorbance (0.0 to 0.02) against distilled water. When β-sitosterol (or stigmasterol) was used instead of cholesterol, the control assay exhibited a linear change in absorbance at 240 nm and 600 nm owing to the increasing turbidity of the sterol (Fig. 6). This change in absorbance was subtracted from that of the assays for estimating the enzymatic activities.

The ABTS assay and the UV absorption method are reproducible with good precision. Two series of enzyme measurements were performed in parallel by these two methods (Table 1). We found a coefficient of variation of 2.3 to 2.4% and 3.2 to 3.7% for the former and the latter method, respectively. The calculated regression line and correlation coefficient (r) indicated excellent agreement between the method of Allain *et al.* (1) and the present coupled assay ($r > 0.998$).

The present coupled assay appeared to be better than the AAP assay in terms of simplicity and the use of nontoxic material. Phenol and AAP used in the reference procedure are toxic and harmful. The ABTS assay is 3.4 times more sensitive than the AAP assay (ratio of the molecular absorption coefficients: $1.82 \times 10^4/5.33 \times 10^3$).

Methods described in the literature for monitoring microbial cholesterol oxidase production during cell fermentation are indirect procedures. They are based upon incubation of whole cells with colloidal suspensions of cholesterol, followed by extraction and determination of either the reaction product, 4-cholesten-3-one, or the remaining substrate. This determination has been made by Liebermann-Burchard assay (for cholesterol) (4), UV absorption (19), or gas chromatography (Kreit, J., Lefebvre, G., and Germain, P., manuscript in preparation). Proce-

TABLE 1

Reproducibility of Cell-Free Cholesterol Oxidase Measurements[a]

Methods	Experiment number	Means		Standard deviation		Coefficient of variation (CV) (%)
		Abs. units	EU/mg	Abs. units	EU/mg	
UV assay	1	0.347	0.881	0.013	0.032	3.7
	2	0.682	0.866	0.022	0.027	3.2
ABTS assay	1	0.432	0.844	0.010	0.019	2.3
	2	0.858	0.838	0.021	0.020	2.4

[a]Ten determinations were made in each experiment under standard conditions; 0.02 mL (18 μg protein) from an enzymic Triton extract was used per assay. All reagents were independently added into the spectrophotometer cuvette; the reaction lasted 5 min in Experiment 1 and 10 min in Experiment 2. EU, enzyme unit.

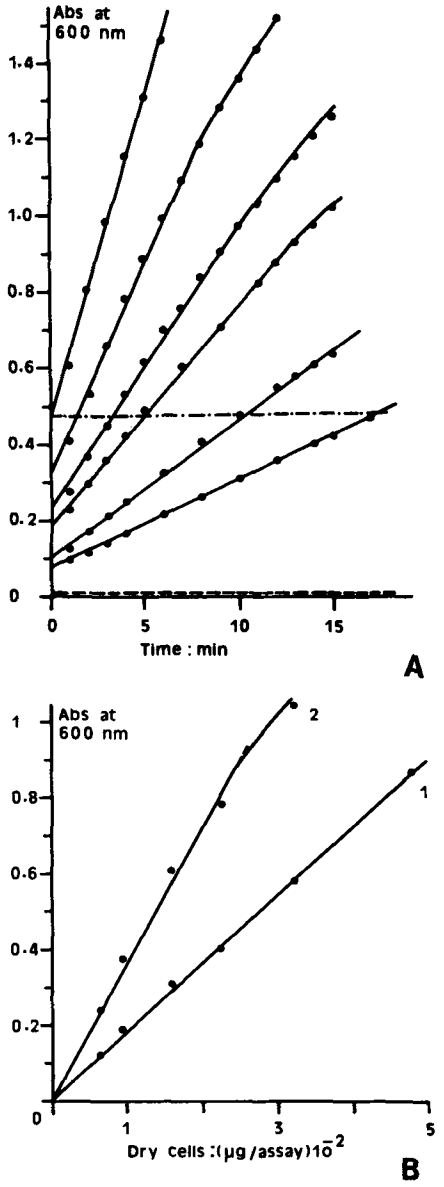

FIG. 7. Measurement of cell-bound cholesterol oxidase activity. a: A cell suspension (cell dry weight: 12.8 mg/mL) was appropriately diluted and then aliquots were assayed by the ABTS assay method. The control was either without cholesterol (—·—·—) or without cells (———). Absorbance was monitored against distilled water. b: Data derived from a. The enzymic activity is per assay of 5 min (1) and of 10 min (2).

dures based on extraction of cholesterol oxidase from cells and assay of the cell-free enzyme have also been reported (6,15). All these methods consist of several steps involving complex experimental conditions; to our knowledge, no suitable direct procedure exists for measuring cell-bound cholesterol oxidase.

In the nocardioform bacteria, cholesterol oxidase has an amphipathic structure consisting of an enzymicly active portion and a hydrophobic portion which allows incorporation of the enzyme into the bacterial cell membrane (5 and Kreit, J., Lefebvre, G., and Germain, P., manuscript in preparation). The enzymicly active site is free in the membrane outer compartment and can exhibit full activity. Direct measurement of the cell-bound enzyme is then possible provided reagents can diffuse inside the microbial cell wall to the enzymicly active site, and again outside the wall after catalysis. This is the case with the present coupled assay for measuring cell-bound cholesterol oxidase of *Rhodococcus* species. When portions of the cell culture or cell suspension were assayed, the rate of enzymic activity was proportional to the cell concentration in the test medium (Fig. 7). Turbidity caused by cells in the assay mixture is acceptable up to 0.5 absorbance unit. The accuracy of the present colorimetric method was investigated using whole cells of the bacterium (Table 2). We found a precision (CV—coefficient of variation) of 3.6% and 3.9% for enzyme activities (in abs. units) with a mean value of 0.585 and 0.311, respectively.

Different enzymic fractions were assayed using the two methods with either cholesterol or β-sitosterol as substrate (Table 3). The enzyme activity values obtained with the ABTS assay correlate well with those obtained in parallel by the UV absorption assay. Cholesterol oxidase in conjugated fractions was measured by the ABTS assay, and recovery of the enzyme was calculated for each fraction tested. As shown in Table 4, the final amount of enzyme in the extracts (or the cell suspension) plus the growth filtrate is comparatively similar to that of the microbe culture from which the fractions were prepared. Assuming that preparation of the fractions was achieved without loss of enzyme activity, the data demonstrate the validity of the coupled assay to measure cell-bound cholesterol oxidase as well as the cell-free enzyme.

Based on kinetic studies, the Michaelis-Menten constant (K_m) and the activation energy (E_a) of the oxidase

TABLE 2

Precision of Cell-Bound Cholesterol Oxidase Measurements by the ABTS Assay Method[a]

Cell fractions	Means		Standard deviation		Coefficient of variation (CV) (%)
	Abs. units	EU/mL	Abs. units	EU/mL	
Cell suspension	0.585	0.823	0.021	0.030	3.6
Complete culture	0.311	0.273	0.012	0.011	3.9

[a]Ten determinations were made for each cell fraction under standard conditions. Cell suspension as described in Figure 7, 0.32 mg dry cells (in 0.1 mL) was used per assay. Complete culture: diluted 5 times; 0.2 mL of the dilution was used per assay. Reaction time, 5 min. EU, enzyme unit.

TABLE 3

Cholesterol Oxidase Measurements by the ABTS Assay and the UV Absorption Method[a]

Experiment number	Substrate	Cholesterol oxidase activity (EU/mg)	
		ABTS assay	UV absorption
1	cholesterol	0.871	0.906
2	cholesterol	0.919	0.878
	β-sitosterol	0.592	0.549
3	cholesterol	1.054	1.130
4	cholesterol	0.045	0.051
	β-sitosterol	0.031	0.034

[a]In the standardized assay medium, 0.98 μmole of either cholesterol or β-sitosterol was used. Experiments were performed with different Triton extracts (1,2), a concentrated fraction of a Triton extract (3), and a growth filtrate (4); ε_m of 4-sitosten-3-one at 240 nm was considered to be the same as that of 4-cholesten-3-one. EU, enzyme unit.

TABLE 4

Recovery of Cholesterol Oxidase in Conjugated Fractions As Evaluated by the ABTS Assay Method[a]

Fractions	Volume (mL)		Cholesterol oxidase activity				Recovery (%)	
			EU/mL		Total (EU)			
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Culture	200	180	0.230	0.306	46.0	55.1	100	100
Growth filtrate	192	174	0.027	0.052	5.2	9.0	11	16
Cell suspension	50	30	0.870	1.500	43.5	45.0	95	82
Extract 1	46	28	0.840	1.350	38.6	37.8	89	84
Extract 2	46	28	0.132	0.180	6.1	5.0	14	11

[a]Recovery of the enzyme in the extract fractions was calculated relative to that of the cell suspension. Two Triton treatments of cell suspensions were generally sufficient for cholesterol oxidase total extraction. EU, enzyme unit.

catalyzing cholesterol were determined by the ABTS assay and, for comparison, by the UV absorption method. In both cases, K_m was about 2×10^{-5}M (from Lineweaver-Burk plots) at pH 7.0 and 30°C and E_a (calculated according to the Arrhenius equation) was about 11 Kcal/mole at pH 7.0. These kinetic data further confirmed the validity of the coupled assay for studying cholesterol oxidase activity.

The present paper represents the first report describing the use of ABTS as chromogen for cholesterol oxidase measurement. The method affords a simple and highly sensitive assay to measure cell-free and cell-bound cholesterol oxidase activities. In terms of efficiency and speed, this assay is expected to be particularly useful for monitoring microbial production of cholesterol oxidase during cell fermentation. Furthermore, the assay represents a novel approach for measuring sterols in biological fluids.

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COMMUNICATION

The Effect of Amino Acids on Choline Uptake and Phosphatidylcholine Biosynthesis in Mammalian Hearts

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The uptake of choline in mammalian hearts in the presence of amino acids was examined. Isolated hamster, guinea pig, rat and rabbit hearts were perfused with labeled choline in the presence and absence of amino acids. Neutral amino acids enhanced choline uptake in the hamster heart, but not in the guinea pig, rat and rabbit hearts. Phosphatidylcholine biosynthesis in these hearts was not affected by the presence of amino acids. Choline uptake in the hamster myocytes was also enhanced by neutral amino acid. The enhancement of choline uptake suggests a direct interaction between the amino acid and the transport of choline into the myocardiac cells. The different responses in choline uptake to neutral amino acids indicate that the regulation of choline uptake in the hearts may be different between mammalian species.
Lipids 27, 466–469 (1992).

Phosphatidylcholine is the major phospholipid in mammalian tissues (1). The principal route for its biosynthesis in the heart and isolated cardiac myocytes is the cytidine-5′-diphosphocholine (CDPcholine) pathway (2–4). Exogenous choline is actively transported into the heart, and rapidly phosphorylated into phosphocholine. Phosphocholine is converted to CDPcholine by the action of cytidine-5′-triphosphate:phosphocholine cytidylyltransferase. The CDPcholine produced is condensed with 1,2-diacylglycerol for the formation of phosphatidylcholine (3). Although the control of the CDPcholine pathway is usually mediated *via* the rate-limiting step of the pathway catalyzed by the cytidylyltransferase, modulation of choline uptake may contribute to the overall regulation of phosphatidylcholine biosynthesis (3).

A number of compounds including hemicholinium-3 (5,6), benzylcholine and chlorocholine (7) have been identified as potent inhibitors of choline uptake. The influence of amino acids on choline uptake and phosphatidylcholine biosynthesis in renal cortical slices has been reported (8). Recently, neutral amino acids were found to enhance choline uptake in the isolated hamster heart, but not to affect phosphatidylcholine biosynthesis (9). However, the ability of neutral amino acids to enhance choline uptake in the hearts of other mammalian species was not studied. Choline in the perfusate was transported across the endothelial cells and the intercellular fluid prior to uptake by the myocardial cells; thus the direct action of amino acid on choline uptake in these cells had not been demonstrated.

In the present report, the effect of amino acids on choline uptake in isolated guinea pig, rat and rabbit hearts were studied and compared with the hamster heart. The ability of the neutral amino acid to enhance choline uptake in isolated hamster and rat myocytes was examined.

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Abbreviations: CDPcholine, cytidine-5′-diphosphocholine; TLC, thin-layer chromatography; CTP, cytidine-5′-triphosphate.

MATERIALS AND METHODS

Materials. Choline iodide, phosphocholine chloride, cytidine 5′-diphosphocholine (CDPcholine), amino acids and all cell culture media were purchased from Sigma Chemical Co. (St. Louis, MO). [Methyl-^{3}H]choline was obtained from NEN division of Dupont (Mississauga, Canada). Thin-layer chromatographic plates (Sil-G25) were a product of Macherey-Nagel (Düren, Germany), and were obtained through Brinkman (Rexdale, Canada). Collagenase (type II) was purchased from Worthington-Miles (Freehold, NJ). Earle's Balanced Salt solution was obtained from Flow Laboratories (Mississauga, Canada). All other chemicals were of the highest grades available and were obtained from the Canlab division of Baxter (Mississauga, Canada).

Methods. Syrian golden hamsters (110 ± 20 g) and guinea pigs (280 ± 20 g) were obtained from Charles River Inc. (Charles River, Canada). Sprague-Dawley rats (230 ± 10 g) and New Zealand White rabbits (1.8 ± 0.5 kg) were obtained from the central animal care unit of the University of Manitoba (Winnipeg, Canada). Hearts were removed from the animals immediately after decapitation, and perfused in Langendorff mode with Krebs-Henseleit buffer containing labeled choline (2). Perfusion time was 60 min at 37°C and the flow rate of the perfusate was 6–8 mL/g heart weight which would produce an aortic pressure of 60–80 mm Hg. The perfusate was saturated with 95% O_2/5% CO_2 and contained 10 μM [methyl-^{3}H]choline (0.35 μCi/mL)/1 mM amino acid. Hearts perfused with labeled choline in the absence of amino acid were used as controls. Subsequent to perfusion, the heart was homogenized in 10 mL $CHCl_3$/CH_3OH (1:1, vol/vol). The homogenate was centrifuged at 2,000 × *g* for 10 min, and the pellet was reextracted twice with the same solvent. An aliquot of the pooled extract was used to determine total choline uptake. Water and chloroform were added to the remainder of the lipid extract in order to reach a ratio of 4:2:3 (vol/vol/vol) for $CHCl_3$/CH_3OH/H_2O. Subsequent to phase separation, the volumes of both upper and lower phases were reduced by evaporation *in vacuo*. An aliquot of the upper phase was analyzed for radioactivity in the choline-containing metabolites by thin-layer chromatography (TLC) with a solvent containing CH_3OH/0.6%-NaCl/NH_4OH (10:10:1, vol/vol/vol). An aliquot of the lower phase was analyzed for radioactivity in the phosphatidylcholine fraction by TLC with a solvent containing $CHCl_3$/CH_3OH/H_2O/CH_3COOH (70:30:4:2, by vol).

Myocytes from the rat heart were isolated by the procedure of Langer *et al.* (10). The viability of the cells was tested by trypan blue exclusion and myogenic activity. Over 90% of the cells in the petri dish were viable and in rod shape. The isolation of myocytes from the hamster

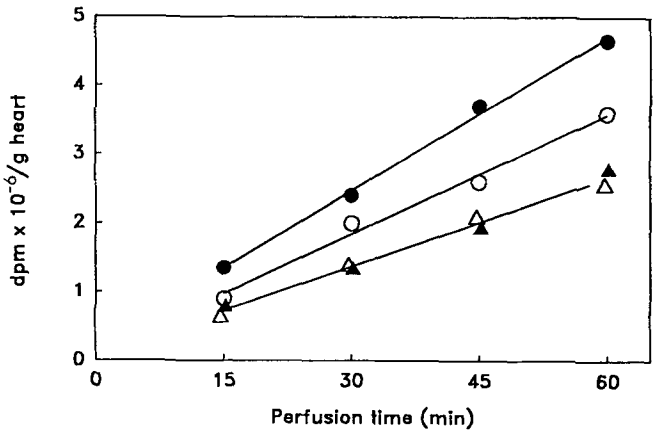

FIG. 1. Time course of choline uptake in hamster and rat hearts in the presence of L-alanine. Hamster (●,○) and rat (▲,△) hearts were perfused in Krebs-Henseleit buffer containing 10 μM labeled choline in the presence (filled symbols) and absence (open symbols) of 1 mM L-alanine for 15–60 min. Total choline uptake was determined in the tissue extract after perfusion. Each time point represents the mean of three separate experiments.

heart required a slight modification of the preceding method. Briefly, the hamster heart was perfused with a modified Jokliks-MEM buffer where sodium bicarbonate was replaced by potassium bicarbonate. The heart was perfused in the modified Jokliks-MEM buffer containing 2.5 mg/mL collagenase for 15 min. Digested tissues were suspended in the same buffer and gently agitated to dislodge the cells. Free myocytes were separated from the other cells by sedimentation. The cells were washed with the modified Jokliks-MEM buffer and incubated with the same buffer for 20 min. The cells were washed with M199 buffer, and subsequently with M199 buffer containing 5% fetal calf serum. The cell suspension was distributed to ten 60-mm petri dishes and the dishes were incubated for 60 min at 37° to allow the attachment of myocytes onto the dish. Over 90% of the cells in the dish were viable.

In the labeling study, myocytes were incubated with Earle's buffer containing 0.1 μM [methyl-^{3}H]choline (2.5 μCi/mL) for 60 min. Subsequently, the cells were washed three times with ice-cooled Earle's buffer. After the wash, 2 mL of 50% methanol was added, and the precipitate was removed by centrifugation. An aliquot of the extract was used for the determination of total uptake. Chloroform and water were added to the extract to cause phase separation. An aliquot of the lower phase was analyzed for radioactivity in the phosphatidylcholine fraction by TLC.

Protein content was determined by the method of Lowry *et al.* (11). Radioactivity was determined by scintillation counting using channels' ratio calibration method. Student's *t*-test was used for statistical analysis.

RESULTS

The time course of choline uptake by hamster and rat hearts in the presence of 1 mM L-alanine was examined. Hearts were perfused with 10 μM labeled choline (0.35 μCi/mL) in Krebs-Henseleit buffer for 15–60 min at 37°C. Subsequent to perfusion, the amounts of radioactivity in the tissue extracts were determined. As depicted in Figure 1, choline uptake was enhanced at all time points by L-alanine in the hamster heart but not in the rat heart. In a separate study, choline uptake in guinea pig and rabbit hearts were not enhanced by 1 mM L-alanine at 30 and 60 min of perfusion.

The effect of other amino acids on choline uptake in hamster, rat, guinea pig and rabbit hearts was also studied. In the presence of 1 mM L-aspartate, no change in choline uptake was observed in all hearts when compared with the control (Table 1). In the presence of 1 mM glycine or L-alanine, the uptake of choline was elevated in the hamster heart but not in guinea pig, rat or rabbit hearts. The choline uptake in the guinea pig, rat or rabbit hearts was not affected when the level of L-lysine, L-aspartate, L-alanine or glycine was elevated to 5 mM.

The effect of amino acids on the labeling of phosphatidylcholine was examined. Subsequent to perfusion, the phosphatidylcholine fraction was isolated from the tissue extract by TLC, and the amount of radioactivity in this fraction was determined (Table 2). As expected, no significant change was observed in the labelling of phosphatidylcholine in the guinea pig, rat or rabbit heart when amino acid was present in the perfusate. Although choline uptake was enhanced in the hamster heart perfused with neutral amino acids, there was no corresponding increase in the labeling of phosphatidylcholine. Analysis of the choline-containing metabolites revealed that the metabolites in the CDPcholine pathway were not affected by L-alanine in guinea pig, rat or rabbit hearts. An accumulation in the labeling of phosphocholine was found in the hamster heart perfused with L-alanine (Table 3). The increase in the labeling of phosphocholine quantitatively

TABLE 1

The Effect of Amino Acids on Choline Uptake in Mammalian Hearts[a]

Amino acid (1 mM)	Hamster heart	Guinea pig heart	Rat heart	Rabbit heart
	(dpm × 10^{-6}/g heart wet weight)			
Control	3.60 ± 0.28 (6)	2.12 ± 0.19 (7)	2.57 ± 0.30 (8)	2.48 ± 0.34 (4)
Glycine	4.72 ± 0.17 (4)[b]	2.19 ± 0.18 (4)	2.75 ± 0.22 (5)	—
L-Alanine	4.66 ± 0.21 (4)[b]	1.90 ± 0.13 (4)	2.76 ± 0.34 (6)	2.31 ± 0.27 (4)
L-Lysine	3.54 ± 0.33 (4)	2.06 ± 0.06 (3)	2.33 ± 0.32 (4)	2.05 ± 0.33 (3)
L-Aspartate	3.47 ± 0.25 (4)	2.09 ± 0.18 (4)	2.79 ± 0.23 (4)	2.38 ± 0.27 (3)

[a]Hamster, guinea pig, rat and rabbit hearts were perfused with 10 μM labelled choline (0.35 μCi/mL) for 60 min, and the amounts of radioactivity in the tissue extracts after perfusion were determined. The values represent the mean ± standard deviation (number of experiments in parentheses).

[b]$P > 0.05$ when compared with control.

TABLE 2

The Effect of Amino Acids on the Incorporation of Labelled Choline into Phosphatidylcholine in Mammalian Hearts[a]

Amino acid (1 mM)	Hamster heart	Guinea pig heart	Rat heart	Rabbit heart
	(dpm × 10^{-5}/g heart wet weight)			
Control	7.75 ± 0.63 (6)	5.47 ± 1.25 (7)	4.00 ± 0.80 (8)	4.12 ± 0.91 (4)
Glycine	7.47 ± 0.54 (4)	6.10 ± 0.58 (4)	3.71 ± 0.83 (5)	—
L-Alanine	7.89 ± 0.32 (4)	4.79 ± 0.71 (4)	4.18 ± 1.37 (6)	4.86 ± 0.47 (4)
L-Lysine	8.04 ± 0.66 (4)	5.51 ± 0.46 (3)	4.90 ± 0.86 (4)	4.41 ± 0.65 (3)
L-Aspartate	7.33 ± 0.71 (4)	4.05 ± 1.18 (4)	3.95 ± 0.24 (4)	4.04 ± 0.54 (3)

[a]Hamster, guinea pig, rat and rabbit hearts were perfused with 10 μM labelled choline (0.35 μCi/mL) for 60 min, and the amounts of radioactivity incorporated into the phosphatidylcholine fractions after perfusion were determined. The values represent the mean ± standard deviation (number of experiments in parentheses).

TABLE 3

The Effect of L-Alanine on the Labelling of Choline-Containing Metabolites in Mammalian Hearts[a]

	Choline	Phosphocholine	CDP-choline
	(dpm × 10^{-5}/g heart wet weight)		
Hamster			
Control	2.47 ± 0.31	25.4 ± 2.9	0.91 ± 0.13
+ L-alanine	2.33 ± 0.30	35.3 ± 2.7[b]	1.00 ± 0.05
Guinea Pig			
Control	2.18 ± 0.68	7.53 ± 1.1	1.08 ± 0.88
+ L-alanine	2.33 ± 0.73	8.40 ± 1.6	0.81 ± 0.35
Rat			
Control	2.73 ± 0.28	14.6 ± 2.7	1.37 ± 0.70
+ L-alanine	2.43 ± 0.48	13.0 ± 3.4	1.26 ± 0.57
Rabbit			
Control	1.77 ± 0.43	11.4 ± 3.0	0.69 ± 0.30
+ L-alanine	1.64 ± 0.22	12.4 ± 1.6	0.84 ± 0.16

[a]Hearts were perfused with labeled choline in the absence and presence of 1 mM L-alanine as described in Table 1. The radioactivities associated with the choline-containing metabolites were determined. The values represent the mean ± standard deviation of four separate experiments.

[b]$P < 0.05$ when compared with control.

TABLE 4

The Effect of L-Alanine on Choline Uptake and Choline Incorporation into Phosphatidylcholine in Isolated Myocytes from Hamster and Rat Hearts[a]

Myocytes	Choline uptake	Phosphatidylcholine
	(dpm × 10^{-3}/μg protein)	
Hamster (batch A)		
Control	1.13 ± 0.11 (4)	0.29 ± 0.04 (4)
+ L-alanine	1.58 ± 0.15 (4)[b]	0.32 ± 0.06 (4)
Hamster (batch B)		
Control	0.82 ± 0.19 (4)	0.28 ± 0.03 (4)
+ L-alanine	1.60 ± 0.13 (4)[b]	0.29 ± 0.05 (4)
Rat		
Control	0.40 ± 0.01 (4)	0.10 ± 0.01 (4)
+ L-alanine	0.38 ± 0.03 (4)	0.10 ± 0.02 (4)

[a]Myocytes from hamster and rat hearts were incubated with 0.1 μM of labelled choline (2 μCi/mL) for 60 min. The radioactivities in the cell extracts and in phosphatidylcholine after the incubation were determined. The values represent the mean ± standard deviation (number of determinations in parentheses).

[b]$P < 0.05$ when compared with control.

accounted for the increase in labelled choline uptake in the presence of the neutral amino acid.

The ability of neutral amino acid to enhance choline uptake in myocytes was investigated. Myocytes were isolated from hamster and rat hearts by collagenase digestion, and incubated with 0.1 μM labeled choline (2 μCi/mL) for 60 min in the presence of L-alanine. Since the viability of the hamster myocytes might not be identical from one batch of cells to another, the variation might affect the ability of the cells to take up choline. The results obtained from two separate batches are shown in Table 4. Choline uptake in hamster myocytes was enhanced by L-alanine in both batches. Analysis of the choline-containing metabolites revealed that their distribution pattern (the accumulation of label in the phosphocholine fraction) was similar to that obtained from the isolated hamster heart. As a control study, the effect of L-alanine on choline uptake in rat myocytes was investigated. The presence of L-alanine did not enhance the uptake of choline in rat myocytes.

DISCUSSION

The uptake of choline by the low-affinity Na^+-independent transport system in non-neural cells has been documented (12). In the hamster heart, the uptake of choline follows Michaelis-Menten kinetics and appears to consist of a single uptake system (2). We have shown earlier that the choline uptake in the hamster heart was enhanced by neutral amino acids (9). We have also demonstrated in the same study that the presence of more than one neutral amino acid did not produce a synergistic effect on the choline uptake (9). The present study suggests that the enhancement of choline uptake by amino acids may be limited to the hamster heart. The inability of neutral amino acids to modulate choline uptake in the guinea pig, rat and rabbit hearts was not caused by the duration of perfusion or amino acid concentration and implies that the regulation of the uptake mechanism is not the same between mammalian species. Interestingly, the hamster heart (and myocytes) also displays a higher rate of choline uptake than hearts from the other animals. The reason for the higher rate of choline uptake in the hamster heart is not known.

Since choline uptake in guinea pig, rat and rabbit hearts was not affected by the presence of amino acids, the rate of phosphatidylcholine biosynthesis in these hearts should not be affected. This postulation was confirmed by the analysis of labeling in phosphatidylcholine and choline-containing metabolites. The ability of the hamster heart to maintain phosphatidylcholine biosynthesis during enhanced choline uptake was documented in previous studies (9). The accumulation of labelled phosphocholine in the hamster heart and in the hamster myocytes clearly reflects the ability of the rate-limiting step of the CDPcholine pathway to regulate phosphatidylcholine biosynthesis (3). At present, the exact mechanism for the modulation of choline uptake by neutral amino acids in the hamster heart is not known. One explanation is that neutral amino acid facilitates the transport of choline across the endothelial wall of the blood vessels and causes an increase in choline concentration in the intercellular fluid. The higher concentration of choline in the intercellular fluid may cause an increase in choline uptake by the myocardial cells. However, the ability of neutral amino acids to enhance choline uptake in the hamster myocytes eliminates this possibility and suggests that such an enhancement is a result of the direct modulation of the choline transport site by amino acids.

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Changes in Lipid Content, Fatty Acid Composition and Lipoprotein Lipase Activity in Dry Goat Omental Adipose Tissue According to Tissue Site

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Water and lipid contents, fatty acid distribution, and lipoprotein lipase activity were determined in 11 samples taken from the *Omentum Majus* of five dry Alpine goats. Samples were chosen to standardize sampling sites using geometric guide marks representative of different adipose tissue sites. Sample location explained between 20% and 30% of the total variance in water and lipid contents and in lipoprotein lipase activity, and from 5.5% to 45.4% of the total variance in fatty acid distribution. Increased sample thickness was associated with an increase in lipid content and in saturated fatty acid percentages. Samples taken in proximity of the omentum tissue attached to the rumen and abomasum had the highest content. We furthermore found that the levels of 18:2n-6, 18:1n-7, and of branched chain fatty acids were high close to a pila of the rumen which also corresponded to high lipoprotein lipase activity. Concomitant high levels of 16:1n-7, 17:1n-8, and 18:1n-9 may reflect high levels of Δ9 desaturase activity. *Lipids 27*, 470–473 (1992).

At the onset of lactation, high-producing dairy females are in a negative energy balance. They mobilize lipids from fat deposits, especially from visceral adipose tissue (1,2). There are great differences between animals, within the same species, the same breed, and even on the same diet, in the ability of an animal to mobilize and restore fat deposits.

The composition and metabolic characteristics of most adipose deposits are well documented in goats (3–6), but there is a general lack of information on the variations that exist within each deposit. In pig and cattle subcutaneous adipose tissue, there is an increase in unsaturation and a decrease in lipogenic enzyme activities with increased proximity to the skin (7–10). In goats, subcutaneous adipose tissue is scarce, but visceral adipose deposits are relatively large (3,11).

The purpose of this study was to compare lipid contents, fatty acid compositions, and lipoprotein lipase activity at different locations of omental tissue in dry goats in order to gain a better understanding of the representativeness of a sample in respect to tissue site.

MATERIALS AND METHODS

Animals and diets. Five dry Alpine does were given free access to lucerne hay, plus 400 g of a cereal based concentrate. Goats, at approximately 4.4 (SD = 1.8) years of age, were slaughtered after an overnight fast between 9:00 a.m. and 10:00 a.m. by stunning and bleeding. Mean live weight was 57.9 kg (SD = 10.6). Immediately after slaughter, the *Omentum Majus*, the perirenal adipose tissue and the mesenteric adipose tissue were removed and weighed. The empty body weight of goats was determined as live weight minus the weight of faeces, digesta, and urine. From each goat, 11 samples were taken from the *Omentum Majus*. These samples were chosen to be representative of the different parts of the adipose tissue. In order to standardize the sampling site for each goat, geometric guide marks were used (Fig. 1). Samples weighing between 5 and 10 g were taken and immediately placed into weighed bags and frozen at −50°C until further analysis.

Chemical analysis. Water and lipid contents were determined ater 48-h freeze-drying and by reflux extraction with hexane (12) using a Soxtec HT extractor (Perstorp, Bezons, France), respectively.

Fatty acid analysis. Fatty acids were butylated in the presence of 3% hydrochloric acid in butanol (vol/vol) as previously described (11). After elimination of excess butanol by washing with distilled water, the fatty acid butyl esters were analyzed by gas-liquid chromatography on a model 300 C Girdel apparatus (Delsi-Nermag, Argenteuil, France). The glass capillary column, 50 m × 0.25 mm, i.d. was coated with carbowax-20 M. The capillary tubing was dynamically coated with the liquid phase in methylene chloride, 20% w/v, using a 2-cm plug of high purity mercury. Peak areas were measured using a Delsi model Icap 10-5 computing integrator (Delsi-Nermag). Peaks were identified on the basis of their equivalent chain lengths (13) as determined by interpolation between two consecutive even-straight-chain saturated fatty acids and compared with those of standards (Sigma, St. Louis, MO) analyzed under the same conditions as well as based on literature values (14–18). Fatty acids of all samples were analyzed as such and following hydrogenation. Hydrogenation was done according to Poukka *et al.* (19) using 1 to 10 mg of butyl esters dissolved, in a conical tube, using 8 mL of butanol to which 20 mg of Adams' platinum oxide catalyst (Touzart et Matignon, Vitry-sur-Seine, France) was added. A slight hydrogen-stream was passed over the solution for 1 h.

Enzyme assay. For each sample, 1 g of frozen crude tissue was homogenized in cold (4°C) heparinized ammoniacal buffer (pH 8.30 at 20°C) using a Sorvall (Norwalk, CT) omni-mixer. Lipoprotein lipase activity was measured in the supernatant according to Chilliard (20).

Statistical analysis. The data were analyzed using the general linear models (GLM) and the discriminant analysis (DISCRIM) procedures provided by the Statistical Analysis System (SAS) (21). Analysis of variance of each dependent variable was performed with the main effect model where the 5 goats and the 11 locations of the samples represented two main factors of variations. The comparison between the location of the samples was performed by the multiple comparison procedure using

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Abbreviations: BCFA, branched-chain saturated fatty acids; DISCRIM, discriminant analysis; ESFA, even-numbered saturated fatty acids; GLM, general linear models; LPL, lipoprotein lipase; LSD, least significant difference; MUFA, monounsaturated fatty acids; OM, *Omentum Majus*; OSFA, odd-numbered saturated fatty acids; SAS, Statistical Analysis System.

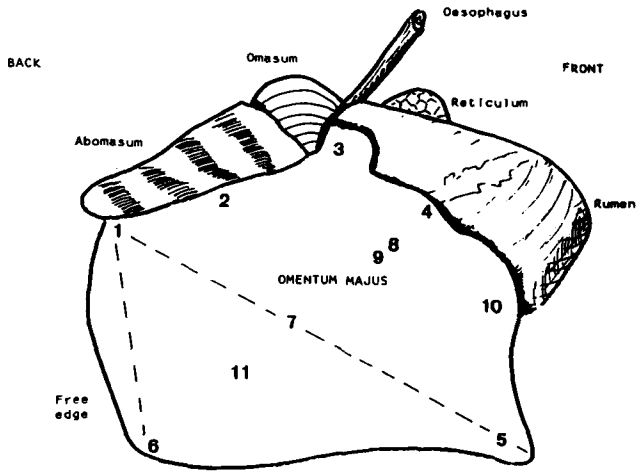

FIG. 1. Location of the samples taken from the *Omentum Majus* (OM) of dry Alpine goats. (1) At the bottom of the abomasum, near the pylorus; (2) near the attached part of the OM, on the *curvature dorsalis* of the abomasum; (3) near the upper part of the abomasum; (4) in front of the spleen, near the *pila longitudinalis* and the *sulcus caudalis*; (5) at the end of the free side of the OM, on the opposite side of sample (1), at the point near the *saccus caecus caudoventralis* of the rumen; (6) at the end of the free side of the OM, on the opposite side of sample (3), near the jejunum; (7) in the middle of the diagonal line between sample (1) and sample (5); (8) between sample (4) and sample (7), on a fatty nodule; (9) between sample (4) and sample (7), on a very thin part of the OM; (10) between sample (4) and sample (5), on the cranial part of the OM, near its connection to the rumen; (11) between sample (6) and sample (7), in a thin area including a blood vessel.

t-test equivalent to Fisher's least-significant difference. The comparisons between the sample locations based on their fatty acid profile were evaluated by discriminant analysis using Mahalanobis distances.

RESULTS

In the five dry goats, the weight of the visceral adipose tissues represented 14.8% (SD = 2.2%) of the empty body weight. Omental tissue was much heavier than the other visceral adipose tissues, representing 48.8% (SD = 5.4%) of the total weight of the three visceral adipose tissues. The coefficient of variation of the omental tissue weight was lower than that of the other visceral adipose tissues (10.8, 22.9 and 30.3%, respectively, for omental, perirenal and mesenteric adipose tissues).

The means of water and of lipid contents, lipoprotein lipase (LPL) activity, and fatty acid percentages for the 55 analyzed samples are reported in Table 1. The means of the 11 samples from each goat omental tissue varied from 91.2% (w/w) to 94.2% (w/w) in lipid content. The higher the lipid content, the smaller were the variations between samples from different sites.

Each determined component of goat omental tissue significantly differed between sample location (Table 2). For water and lipid contents, the analyzed samples could be divided into at least three groups. The group containing samples 4, 9 and 11 was characterized by the highest water content (7.0% w/w) and the lowest lipid content (91.2% w/w) (Table 2, and Fig. 2). The other extreme was represented by samples 2, 3 and 10 which showed the lowest water content (4.6% w/w) and the highest lipid content (94.2% w/w).

LPL activity was high in sample 4 (40 nmol · min^{-1} · g^{-1}) and to a lesser extent in sample 10 (25 nmol · min^{-1} · g^{-1}). LPL activity was the lowest in sample 2 (12 nmol · min^{-1} · g^{-1}).

Total variance with location for fatty acid percentages ranged from 5.5% for 17:0 to 45.4% for 16:1n-7 (Table 1). Variance for 16:1n-7 by location was much greater than variance between goats. Both sources of variations contributed almost equally to the variances observed for

TABLE 1

Between-Goat and Between-Location Sample Variations of Water and Fatty Acid Contents and of LPL Activity in Dry Goat Omental Adipose Tissue

	$\bar{X}$	SD	% of Variance on account of location	% of Variance on account of animal
Water %[a]	5.6	2.03	23.2	32.5
Lipid %[a]	92.9	2.40	29.0	21.6
LPL[b]	20.6	13.50	26.9	32.4
Fatty acids (% w/w)				
14:0	3.0	0.46	11.6	68.1
16:0	29.9	3.09	7.3	62.1
16:1n-7	1.2	0.31	45.4	6.0
17:0	2.2	0.33	5.5	75.7
17:1n-8	0.6	0.18	29.9	40.5
18:0	27.6	3.55	39.0	27.5
18:1n-9	25.6	4.64	26.4	33.8
18:1n-7	2.3	0.34	6.5	73.9
18:2n-6	0.9	0.60	12.7	27.3
BCFA	1.3	0.23	23.6	23.7
OSFA[c]	2.9	0.22	7.1	70.6
ESFA	60.5	4.11	28.3	29.4
MUFA[d]	30.0	3.83	27.9	30.6

[a]% w/w of crude tissue.
[b]Lipoprotein lipase activity (nmol · min^{-1} · g^{-1} of crude tissue).
[c]Odd-numbered saturated fatty acids.
[d]Monounsaturated fatty acids.

TABLE 2

Changes in Composition and in LPL Activity of Dry Goat Omental Adipose Tissue According to Sample Location

Composition and LPL activity	Sample number[a]											LSD[b]	Location effects[c]
	1	2	3	4	5	6	7	8	9	10	11		
Water %[d]	5.8	4.6	4.7	6.7	5.2	6.2	5.2	4.7	6.8	4.5	7.4	2.01	*
Lipid %[d]	92.8	94.3	94.0	91.3	93.5	91.9	93.8	93.9	91.6	94.3	90.7	2.51	*
LPL[e]	18	12	18	40	15	15	18	23	23	25	21	10.82	*
Fatty acids (% w/w)													
14:0	2.6	3.0	2.8	2.7	3.1	3.0	3.1	3.0	3.1	3.0	3.0	0.31	*
16:0	29.4	31.0	29.8	28.4	31.2	29.5	30.5	29.3	29.6	31.0	29.7	2.54	**
16:1n-7	1.1	1.0	1.0	1.5	1.0	1.3	1.3	1.3	1.6	1.0	1.2	0.32	*
17:0	2.1	2.2	2.3	2.1	2.2	2.1	2.2	2.1	2.2	2.3	2.2	0.21	*
17:1n-8	0.6	0.5	0.6	0.8	0.5	0.6	0.7	0.7	0.8	0.5	0.6	0.14	*
18:0	28.4	29.2	30.4	23.8	29.9	26.9	26.8	26.4	23.9	30.1	27.7	3.04	*
18:1n-9	26.2	23.5	23.1	29.8	22.3	26.5	26.0	28.6	29.0	22.7	25.5	4.34	*
18:1n-7	2.3	2.3	2.4	2.4	2.3	2.3	2.2	2.3	2.2	2.2	2.1	0.22	*
18:2n-6	1.0	0.8	0.8	1.1	0.9	1.3	0.6	1.1	0.7	0.6	1.0	0.69	**
BCFA	1.1	1.2	1.3	1.6	1.3	1.2	1.4	1.4	1.3	1.3	1.6	0.29	*
OSFA	2.8	2.8	3.1	2.8	2.8	2.7	2.9	2.8	2.8	3.0	2.8	0.28	*
ESFA	60.4	63.2	63.0	55.0	64.2	59.4	60.4	58.8	56.6	64.1	60.3	5.26	*
MUFA	30.6	27.6	27.4	34.9	26.3	31.0	30.4	31.2	34.0	26.7	29.7	4.90	*

[a]For sample numbers, see Figure 1.

[b]Least significant difference.

[c]Significant effect of the location of the sample at, *, $P < 0.05$ and **, $P < 0.01$, with t-test using least significant difference of GLM from SAS.

[d]% w/w of crude tissue.

[e]Lipoprotein lipase activity (nmol • $min^{-1} \cdot g^{-1}$ of crude tissue).

18:0, 18:1n-9, and branched-chain saturated fatty acids (BCFA).

Total straight-chain saturated fatty acid levels were significantly higher in samples 10, 5, 3, and 2 than in the other samples. These four samples contained high levels of the major saturated fatty acids 16:0 and 18:0, sample 5 had a low level of 15:0, sample 3 had a low level of 14:0, and sample 2 had low levels of 14:0, 15:0, and 17:0. By contrast, the highest levels of total monounsaturated fatty acids were found in samples 4 and 9 due to high 16:1n-7, 17:1n-8, and 18:1n-9 levels. The percentages of the other monoenoic fatty acids, for example, 16:1n-9 and 18:1n-7, were elevated in sample 4 but not in sample 9. Moreover, sample 4 had the highest BCFA content and a high 18:2n-6 level.

The total-sample correlation coefficient for all omental samples (n = 55) calculated between 18:1n-9 on one hand, and 14:0, 15:0, 16:0, 16:1n-9, 16:1n-7, 17:1n-8, 18:0, and 18:2n-6 levels on the other hand, were highly significant (−0.583, −0.595, −0.683, +0.491, +0.710, +0.856, −0.820, and +0.633, respectively). The correlation between 18:1n-9 and 18:1n-7 was very weak (+0.197). Overall, unsaturated fatty acid percentages were positively correlated to LPL activity (+0.418, +0.392, +0.579, +0.497, +0.313, and +0.289 for 16:1n-9, 16:1n-7, 17:1n-8, 18:1n-9, 18:1n-7, and 18:2n-6, respectively; $P < 0.05$). By contrast even-numbered saturated fatty acids (ESFA) levels were negatively correlated to LPL (−0.398, −0.560, and −0.319, respectively, for 14:0, 16:0, and 18:0). The mean thickness of the samples was strongly correlated with water and lipid contents, and the levels of 16:1n-7, 17:1n-8, 18:0, and 18:1n-9 (−0.581, +0.558, −0.479, −0.401, +0.548, and −0.483, respectively).

In regard to the fatty acid profile of each sample location, most samples could be pooled with neighboring ones due to their similar profiles, although samples 4, 9, and 11 were quite dissimilar.

DISCUSSION

Variations in composition within the omentum have not been reported previously for goats or other domestic animals. Observed variations in water content, in lipid content, in LPL activity, and in fatty acid composition between samples from different locations appear to be characteristic for the tissue site. It also seems that the thickness of the sample relates to variations in composition as the three thinner samples had the lowest lipid and saturated fatty acid contents. Samples in close proximity to the attachment site to the rumen and abomasum had increased lipid contents and decreased water contents in comparison to samples distant from the attachment site. Sample 4 was an exception with its high water content, its low lipid content, and its highest levels of monoenoic fatty acids, BCFA, and 18:2n-6, and its very high LPL activity being twice as high as in other samples. This appears to indicate a high metabolic activity at the sample 4 site. The large amounts of dietary fatty acids, for example 18:2n-6, or of 18:1n-7 which are produced in the rumen during microbial hydrogenation of polyunsaturated fatty acids from the diet (22,23), or BCFA which are derived from lipids of rumen microorganisms, emphasize the importance of lipogenesis by uptake of fatty acids from the bloodstream involving lipoprotein lipase. High levels of 16:1n-7, 17:1n-8, and 18:1n-9 may reflect a very active endogenous Δ9 desaturase. Thick samples such as fatty nodules with a high lipid content and a high level of saturated fatty acids seem to point toward a low metabolic activity (24).

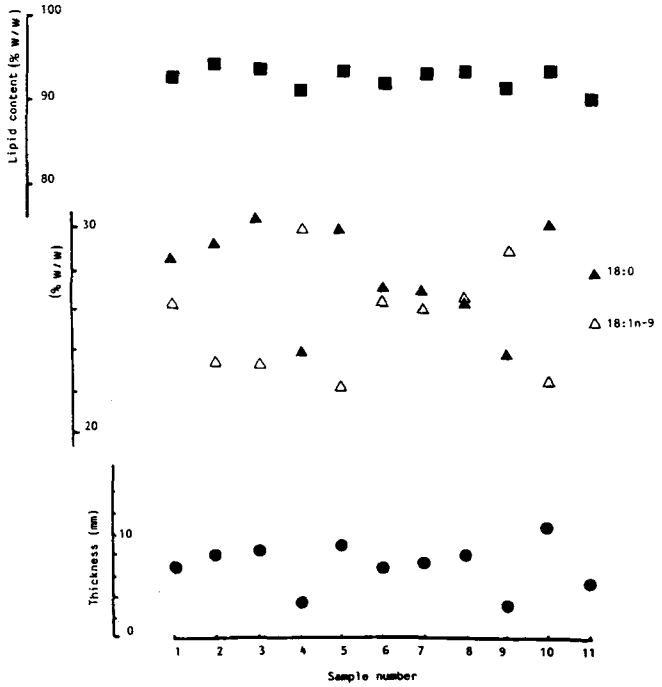

FIG. 2. Variation of some characteristics of goat *Omentum Majus* in dry Alpine goats.

On account of the significant variations observed within the omental tissue, care in the selection of the sampling site appears to be in order. Unless the whole tissue cannot be analyzed, an easily definable sample site should be chosen. The sample which seems to best fit this criterion is sample 5; it is located where the omental tissue forms a sac at its periphery.

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Low-Density Lipoprotein Turnover in Inbred Strains of Rabbits Hypo- or Hyperresponsive to Dietary Cholesterol

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In two inbred strains of rabbits with high or low response of plasma cholesterol to dietary cholesterol, low density lipoprotein (LDL) apolipoprotein (apoLDL) kinetics were determined with the use of a heterologous tracer isolated from a Watanabe heritable hyperlipidemic (WHHL) rabbit. On a diet without added cholesterol, the total clearance of apoLDL (which equals apoLDL production) did not differ significantly between rabbits of both strains. After the feeding of a diet containing 0.1% cholesterol for six weeks, plasma LDL cholesterol, plasma apoLDL and liver cholesterol concentrations rose significantly in the hyperresponsive but not in the hyporesponsive rabbits. Cholesterol feeding depressed the total fractional catabolic rate (FCR) of apoLDL in the hyper- but not in the hyporesponsive rabbits; this was attributed to a decrease of receptor-dependent FCR while receptor-independent FCR was similar in the two strains. On the diet containing cholesterol, the receptor-mediated absolute catabolic rate (ACR) of apoLDL did not differ between hyper- and hyporesponsive rabbits but receptor-independent ACR of apoLDL was higher in hyperresponders. It is concluded that the higher plasma apoLDL levels in hyperresponsive rabbits fed the 0.1% cholesterol diet are caused by a higher production of apoLDL and not by a lower flux of apoLDL through the receptor-mediated pathway.
Lipids 27, 474-477 (1992).

Among humans and various animal species, individuals can differ in their susceptibility to dietary cholesterol (1). Hyporesponders showing only minor changes in their plasma cholesterol concentrations can be discriminated from hyperresponders showing high degrees of cholesterolemia. The underlying cause for this individual variation in response is poorly understood. Studies with inbred rabbits (2,3) indicate that in hyperresponders the efficiency of intestinal cholesterol absorption is increased. After cholesterol feeding, the higher efficiency of cholesterol absorption in hyperresponders leads to increased fluxes of chylomicron-remnant cholesterol into the liver. This might cause higher liver cholesterol concentrations in hyper- than in hyporesponders. The liver is the major organ for low-density lipoprotein (LDL) clearance (4) and there is an inverse relationship between cellular cholesterol concentrations and the number of LDL receptors on the cell membrane (5). Thus, cholesterol feeding might lead to a greater reduction of receptor-mediated LDL clearance in hyper- than in hyporesponders. This in turn would explain the higher degree of hypercholesterolemia in cholesterol-fed hyperresponders compared with their hyporesponsive counterparts. In the present study this idea was put to the test.

MATERIALS AND METHODS

Animals and diets. Rabbits, aged 9-25 mon, of two inbred strains were used. The IIIVO/JU strain has previously been shown to be hyporesponsive and the AX/JU strain to be hyperresponsive to dietary cholesterol (2,6,7). The rabbits had been fed a commercial diet (LKK-20®, Hope Farms, Woerden, The Netherlands) and were housed individually as described (2). Three male and three female rabbits of each of the two strains were either fed a diet without added cholesterol or a high-cholesterol diet. Males and females have been shown to have comparable cholesterolemic responses to cholesterol feeding. A purified diet was formulated so as to keep dietary cholesterol concentration as low as possible. This diet consisted of (g/kg): soybean protein isolate, 102; methionine, 2; dextrose, 64.3; alfalfa meal, 560; molasses, 100; corn oil, 10; coconut fat, 90; $CaCO_3$, 10; NaH_2PO_4, 5; Na_2CO_3, 10.3; $MgCO_3$, 1.4; NaCl, 5; $KHCO_3$, 18; mineral premix, 10; vitamin premix, 12. The composition of the vitamin and mineral premix has been described (7). The purified diet without added cholesterol was fed for four weeks prior to and during the LDL turnover measurement period. To maintain palatability, the high-cholesterol diet was the commercial chow to which 0.1% (w/w) of cholesterol was added. This amount of cholesterol is low compared with the amounts generally used in cholesterol feeding studies with rabbits. In this way, induction of pathological changes would be prevented (8). The high-cholesterol diet was fed for six weeks prior to and during the apoLDL turnover measurement period. Upon analysis, the diet without added cholesterol and the high-cholesterol diet were found to contain 8 and 141 mg of cholesterol/100 g diet, respectively. The rabbits were given 75 g of diet at 9 a.m. each day. This amount was completely consumed within 4 h. Tap water was provided *ad libitum*. The rabbits were allowed to practice cecotrophy.

Isolation, radiolabelling and reductive methylation of LDL. Additional details of the procedures used have been described previously (9). Five days before apoLDL turnover measurement, 70-80 mL of blood was taken by heart puncture from a WHHL rabbit fed the commercial diet. LDL was isolated from serum by sequential ultracentrifugation in a fixed angle rotor at 168,000 × *g* for 22 hr between densities 1.020 and 1.055 as described (9). For administration to rabbits fed the diet without added cholesterol, this LDL was labelled with ^{131}I by a modification (10) of the method of McFarlane (11). For injection into rabbits fed the high-cholesterol diet, a por-

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Abbreviations: ACR, absolute catabolic rate; apo, apolipoprotein; EDTA, ethylenediaminetetraacetic acid, sodium salt; FCR, fractional catabolic rate; LDL, low density lipoproteins; WHHL, Watanabe heritable hyperlipidemic (rabbit).

tion of the LDL was labelled with ^{131}I and another portion with ^{125}I. Subsequently, the ^{125}I-labelled LDL was chemically modified by reductive methylation following a 60-min reaction sequence (12). Methylation of lysine residues has been shown to abolish the binding of LDL to the LDL receptor without affecting non-receptor-mediated processes (10,13). Free iodine was removed by column chromatography on Sephadex G-25 M (columns PD-10, Pharmacia Fine Chemicals, Uppsala, Sweden). As estimated by a colorimetric method based on reaction of 2,4,6-trinitrobenzene-1-sulfonic acid with primary amines (14), 63% of the lysine residues had been methylated. The efficiency of labelling was 50–60%; more than 95% of the radioactivity could be precipitated by 10% trichloroacetic acid and 80–85% by 50% isopropanol.

LDL turnover studies. Two turnover studies were carried out exactly as described (9). One study was done with the animals fed the diet without added cholesterol and another with rabbits fed the high cholesterol diet; the interval between the two studies was 11 wk. ^{131}I-labelled native LDL was diluted with 0.15 M NaCl in the absence (study with rabbits fed the diet without added cholesterol) or presence (study with cholesterol-fed rabbits) of ^{125}I-labelled methyl-LDL and mixed with serum derived from a rabbit of the same strain and fed the same diet as the recipients. This mixture was kept overnight at 4°C after which 1 mL was injected into a marginal ear vein of each of the recipients; the amount of injected radioactivity was 13.8–15.5 μCi (0.51–0.57 MBq) for ^{131}I and 12.8–13.3 μCi (0.47–0.49 MBq) for ^{125}I. The amount of apoLDL injected into the rabbits fed the diet without added cholesterol was less than 0.4% of their estimated apoLDL pool. For the cholesterol-fed animals, this amount was lower than 0.2%. Blood samples drawn 3 min after injection were used to calculate plasma volume by measuring isotope dilution. Blood samples (2–2.5 mL in 4 mg ethylenediaminetetraacetic acid (EDTA) were taken at regular intervals for 72 h. Total plasma radioactivity of ^{131}I and ^{125}I was counted in a Philips PW 4800 automatic gammacounter (Eindhoven, The Netherlands). During blood sampling, the feeding schedule was not changed. Within two hours after taking the last blood samples, the rabbits were killed by administration of pentobarbital, and their livers removed.

The fractional catabolic rate (FCR) was obtained from bi-exponential analysis of the plasma radioactivity decay curves, assuming a simple two-compartment model where plasma LDL equilibrates with an extravascular compartment and where all irreversible loss of apoLDL occurs from the plasma compartment (15,16). Decay of ^{125}I-labelled LDL was used to calculate the receptor-independent FCR. The difference between decay of ^{131}I- and ^{125}I-labelled LDL was taken to calculate the receptor-dependent FCR. The absolute catabolic rate (ACR) was calculated from the FCR and the apoLDL pool size; the apoLDL pool size was obtained from the plasma volume and the apoLDL concentration by multiplication, and expressed relative to body weight. Under steady-state conditions, the ACR equals the production rate.

Analytical methods. After an overnight fast, blood was taken from an ear artery and mixed with EDTA (1 g/L) for lipoprotein analysis. Lipoproteins were isolated by sequential centrifugation in a Kontron fixed angle rotor JFT45 (Hemile, Switzerland) in a Beckman L7/55 ultracentrifuge (Fullerton, CA) at 168,000 × *g* for 20 h at 14°C as described previously (17). LDL was isolated at the density range 1.025–1.055 g/mL. ApoLDL was determined as total protein in LDL.

Total cholesterol was determined with an enzymatic, commercially available test kit (Boehringer, Mannheim, Germany). Protein was determined according to the method described by Lowry *et al.* (18). Liver samples (1–2 g) were homogenized in two volumes of distilled water, and cholesterol was extracted and analyzed according to Abell *et al.* (19).

Statistical analyses. Comparisons between the hypo- and hyperresponsive strain and between animals of the same strain fed either the diet without added cholesterol or the high-cholesterol diet were evaluated by Student's *t*-test. For data with no normal distribution, the Mann-Whitney U test was used. A two-sided *P* value of less than 0.05 was set for statistical significance. Results are expressed as means ± SD.

RESULTS

Plasma lipoproteins and liver cholesterol. Initial plasma total cholesterol concentrations and those after feeding the diet without added cholesterol were higher in the hyporesponsive rabbits (Table 1). When fed the diet without added cholesterol, there were no significant dif-

TABLE 1

Cholesterol and ApoLDL in Plasma and Liver Cholesterol of Hypo- and Hyperresponsive Rabbits Fed Either a Diet Without or with Added Cholesterol[a]

Measure	Diet without added cholesterol		Diet with added (0.1%) cholesterol	
	Hypo-	Hyper-	Hypo-	Hyper-
Plasma total cholesterol (mM)				
Initial	0.98 ± 0.23	0.64 ± 0.22[b]	1.17 ± 0.18	0.57 ± 0.19[b]
Final	2.04 ± 0.65	0.79 ± 0.36[b]	2.16 ± 0.27	4.87 ± 1.27[b,c]
LDL cholesterol (mM)[d]	0.24 ± 0.17	0.20 ± 0.18	0.39 ± 0.15	2.34 ± 1.06[b,c]
ApoLDL (mg/dl)[d]	17.5 ± 6.0	15.4 ± 4.7	20.5 ± 3.4	59.9 ± 23.2[b,c]
Liver cholesterol (μmol/g)	6.4 ± 0.9	9.3 ± 1.4[b]	8.1 ± 1.7	14.7 ± 2.8[b,c]

[a]Results are expressed as means ± SD for 6 animals.

[b]Significant difference between hypo- and hyperresponsive rabbit strain fed the same diet.

[c]Significantly different from rabbits of the same strain but fed the cholesterol-free diet.

[d]At the administration of the tracer(s).

ferences in plasma LDL cholesterol and apoLDL levels between the two strains, whereas liver cholesterol concentrations were significantly higher in the hyperresponders.

Feeding the high-cholesterol diet caused more pronounced increases of plasma total cholesterol concentrations in the hyperresponsive rabbits than in the hyporesponders. The strain difference in response to dietary cholesterol was larger for LDL-cholesterol and apoLDL concentrations in plasma. Cholesterol loading increased group mean liver cholesterol concentrations in both strains, the increase being larger in the hyperresponders.

LDL catabolism. Plasma cholesterol concentrations did not change over the 72 h of measuring apoLDL turnover (data not shown), consistent with reaching a steady state. The size of plasma apoLDL pools in hypo- and hyperresponders fed the diet without added cholesterol was similar (7.8 ± 2.3 *vs.* 8.2 ± 3.4 mg/kg; means ± SD, n = 6). On the high-cholesterol diet, plasma apoLDL pools had significantly increased in both strains, the final value being significantly greater in the hyperresponders (Table 2). On the diet without added cholesterol, total FCR of apoLDL did not differ significantly between the two strains (Fig. 1): 1.63 ± 0.37 and 1.71 ± 0.44 pools/day for hypo- and hyperresponders, respectively (means ± SD, n = 6). Compared with the diet without added cholesterol, the high-cholesterol diet caused a decrease of the mean total FCR of apoLDL in the hyper- but not in the hyporesponders (Table 2). Receptor-independent FCR of apoLDL did not differ significantly between hypo- and hyperresponders fed cholesterol. Receptor-dependent FCR of apoLDL was significantly lower in hyperresponsive rabbits fed the diet containing cholesterol when compared with their hyporesponsive counterparts (Table 2). When fed the diet without added cholesterol, there was no significant difference in ACR of apoLDL between hypo- and hyperresponsive rabbits (12.1 ± 2.1 *vs.* 14.2 ± 7.1 mg/kg/day; means ± SD, n = 6). Dietary cholesterol produced a significantly larger ACR of apoLDL in both rabbit strains, the difference being more pronounced in the hyperresponders.

TABLE 2

Kinetic Data for ApoLDL Turnover in Hypo- and Hyperresponsive Rabbits Fed a Diet with Added (0.1%) Cholesterol[a]

Measure	Hypo-	Hyper-
ApoLDL pool (mg/kg)	12.2 ± 1.9	35.3 ± 13.6[b]
FCR (pools per day)		
Total	1.84 ± 0.32	0.97 ± 0.23[b]
Receptor-independent	0.86 ± 0.37	0.62 ± 0.07
Receptor-dependent	0.97 ± 0.38	0.39 ± 0.20[b]
ACR (mg/kg/day)		
Total	22.2 ± 3.9	31.6 ± 5.9[b]
Receptor-independent	10.2 ± 3.5	20.4 ± 8.8[b]
Receptor-dependent	12.0 ± 5.0	10.6 ± 3.8

[a]Results are expressed as means ± SD for 6 animals.

[b]Significant difference between hypo- and hyperresponsive rabbit strain.

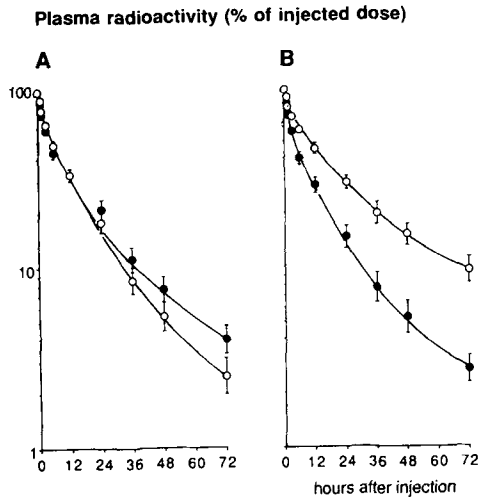

FIG. 1. Disappearance of plasma radioactivity after injection of ^{131}I-labelled native LDL in hypo- and hyperresponsive rabbits fed a diet without (A) or with (B) added (0.1%) cholesterol. Closed symbols: hyporesponders; open symbols; hyperresponders. Values are expressed as means ± SEM.

DISCUSSION

The present study provides further insight into the metabolic differences between hypo- and hyperresponsive inbred rabbits. On the diet without added cholesterol, hyperresponsive rabbits had slightly higher liver cholesterol concentrations but did not differ significantly from hyporesponders as to LDL-cholesterol levels, apoLDL pools and total FCR of apoLDL. On the high-cholesterol diet, LDL-cholesterol, apoLDL and liver cholesterol concentrations became higher in the hyper- than in the hyporesponsive rabbits. Cholesterol feeding depressed total FCR of apoLDL in the hyper- but not in hyporesponsive rabbits. This was due to a decrease of both receptor-dependent and receptor-independent FCR of apoLDL, although the latter was not significantly different between hypo- and hyperresponders.

Since apoLDL pools were significantly higher in the hyperresponders, the observed reduction of the receptor-dependent FCR in this strain cannot be taken as unequivocal evidence that there had been depression of the number of LDL receptors (20). The ACR can provide additional information in this context. Although the calculated ACR values may be biased because a heterologous tracer LDL was used, they may still serve to compare the two rabbit strains. The decreased receptor-dependent FCR of apoLDL in cholesterol-fed hyper- *vs.* hyporesponders, in combination with the increased apoLDL pools in the former, led to receptor-dependent ACR values that were identical for hyper- and hyporesponders fed the high-cholesterol diet. Thus, in cholesterol-fed hypo- and hyperresponders the flux of apoLDL through the receptor-dependent pathway was similar. This could imply that in the two strains the number of LDL receptors was similar while the concentration of circulating LDL particles was sufficiently high to saturate the receptors. Thus, after cholesterol feeding there was no difference in LDL receptor activity in the two strains. Alternatively, it could be proposed that the LDL receptors in cholesterol-fed hyperresponders are less active than in cholesterol-fed hyporesponders. Despite the three-fold larger apoLDL pool in the hyperresponders, the LDL receptors in these rabbits were not able to remove more LDL from the cir-

culation than those in the hyporesponders. This resulted in a lower receptor-mediated FCR of apoLDL in cholesterol-fed hyperresponders. It is possible that the feeding of a higher amount of cholesterol than that used in the present study would induce a clear-cut difference between hypo- and hyperresponders as to depression of LDL receptors.

There was a direct relation between total ACR of apoLDL and plasma apoLDL pools for individual rabbits ($r = 0.87$; $n = 24$; $P < 0.0005$). Thus, it may be concluded that the higher plasma apoLDL concentration in the hyperresponders was caused by a higher production of apoLDL. Since the flux of LDL through the receptor-dependent pathway was not influenced, a new steady state could only be reached by increasing receptor-independent removal of LDL. Indeed, receptor-independent removal (ACR) of apoLDL was higher in the hyper- than in the hyporesponsive rabbits.

We conclude that the earlier reported (2,3) higher efficiency of intestinal cholesterol absorption in hyperresponsive rabbits causes liver cholesterol to increase more markedly in hyper- than hyporesponsive rabbits in response to cholesterol feeding. There was a positive correlation between liver cholesterol concentration and total ACR of apoLDL ($r = 0.71$; $n = 24$; $P < 0.0005$). Possibly, the higher liver cholesterol levels in hyperresponders cause a higher production rate of apoLDL, which accounts for the increase of LDL in plasma. The number of LDL receptors in hypo- and hyperresponders fed a diet with 0.1% cholesterol is similar and, maybe because they are saturated with LDL, the absolute amount of apoLDL taken up by the cells is also similar. Because of the increased LDL concentration in hyperresponders, the amount of apoLDL taken up by the receptor-independent pathway in these animals is increased.

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Effect of 25-Hydroxycholesterol on Cholesteryl Ester Formation in Caco-2 Cells

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Incubation of Caco-2 cells, a human intestinal cell line, with 25-hydroxycholesterol (25-HOC) markedly enhanced cellular cholesteryl ester formation determined by incorporation of [^{14}C]oleic acid into intracellular cholesteryl [^{14}C]oleate. The stimulation by 25-HOC of cholesteryl ester formation was suppressed by staurosporine, a kinase inhibitor, but not by cycloheximide or actinomycin D. The specific activity of microsomal acyl-coenzyme A:cholesterol acyltransferase (ACAT) increased two-fold in cells treated with 10 μM 25-HOC for 5 h. ACAT activity decreased when microsomes were incubated without sodium fluoride, a phosphatase inhibitor, but the decrease in ACAT activity in cells stimulated with 25-HOC was more pronounced. The results suggest that protein phosphorylation may be involved in the stimulation of cholesteryl ester formation by 25-HOC in Caco-2 cells.
Lipids 27, 478–480 (1992).

Oxygenated derivatives of cholesterol, such as 25-hydroxycholesterol (25-HOC) and 7-ketocholesterol, are potent regulators of intracellular cholesterol metabolism (1). In cultured cells, these sterols have been shown to suppress 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and low density lipoprotein receptor activity mainly by the repression of transcriptional expression of the genes through sterol regulatory elements (1).

It has also been reported that oxygenated sterols stimulate the formation of cholesteryl esters in several cultured cell lines including human fibroblasts (2), rat hepatocytes (3) and rat intestinal cells (4). This stimulation is caused by an increase in the activity of microsomal acyl-coenzyme A:cholesterol acyltransferase (ACAT) which catalyzes the formation of long-chain fatty acyl esters of cholesterol. ACAT plays an important role in cholesterol absorption in the intestine and in foam cell formation in arteries; however, the precise mechanism of enhancement of ACAT activity by oxygenated sterols is not clear.

In the present study, the effects of 25-HOC on cholesteryl ester formation were studied in Caco-2 cells, a cell line derived from human intestine, to further explore the mechanism of stimulated cholesteryl ester formation in human intestinal cells. The results confirm that 25-HOC stimulates cholesteryl ester formation in this cell line through enhancement of microsomal ACAT activity. In addition, it appears that protein phosphorylation is involved in this type of stimulation.

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Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; AD, actinomycin D; BSA, bovine serum albumin; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle medium; 25-HOC, 25-hydroxycholesterol; STP, staurosporine; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. [1-^{14}C]Oleic acid, [1-^{14}C]oleoyl-coenzyme A and [4,5-^{3}H]leucine were purchased from New England Nuclear (Boston, MA). Oleic acid, oleoyl-coenzyme A, cholesterol and 25-hydroxycholesterol were purchased from Sigma Chemical Co. (St. Louis, MO). Staurosporine was from Kyowa Hakko, Inc. (Tokyo, Japan). All other chemicals were reagent grade.

Assay of cholesteryl ester formation. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured at 37°C in 95% air/5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal calf serum and antibiotics. Cholesteryl ester formation was estimated according to Brown *et al.* (5). Briefly, cells grown to confluence in 12-well plates (Costar, Cambridge, MA) were washed and treated with or without 25-HOC in DMEM at 37°C for 2 h. Cells were then incubated for 3 h with 100 μM [1-^{14}C]oleic acid (sp. act. 110MBq/mmol) and 20 μM fatty acid-free bovine serum albumin (BSA; the molecular weight of BSA was assumed to be 60,000). Intracellular lipids were extracted with *n*-hexane/2-propanol (3:2, vol/vol) and separated by thin-layer chromatography (TLC). Radioactivity was scanned on the TLC plates with a Berthold Automatic TLC-Linear Analyzer LB285 (Labor Berthold, Wildbad, Germany) to measure incorporation of labeled oleic acid into cholesteryl esters. Cholesterol and 25-HOC were dissolved in ethanol; staurosporine was dissolved in dimethyl sulfoxide. The final concentration of solvents in the medium was 1%.

Assay of [^{3}H]leucine incorporation into cells during cholesteryl ester formation. Cells grown on 12-well plates were incubated for 3 h with [^{14}C]oleic acid, BSA and [^{3}H]leucine (75 KBq, sp. act. 37 GBq/mmol). After lipids were extracted, the residue was washed twice with cold 5% trichloracetic acid and solubilized in 1 mL of 1 N NaOH. [^{3}H]Leucine incorporated into the cells was determined by liquid scintillation counting of the solution after neutralization. Cycloheximide and actinomycin D were dissolved in DMEM.

Assay of ACAT. Caco-2 cells were grown to confluence in 225-cm^2 flasks (Costar), and treated with 10 μM 25-HOC for 5 h. Microsomes were prepared as 100,000 × *g* pellets from cell homogenates by sequential centrifugation. ACAT activity was measured as described (2). After 5 min preincubation at 37°C, microsomes (40 μg of protein) were incubated with 20 μM [^{14}C]oleoyl-CoA (sp. act. 220 Bq/mmol) and 20 μM BSA for 10 min in 154 mM potassium phosphate buffer (pH 7.4), in a total volume of 500 μL. Lipids were extracted with chloroform/methanol (2:1, vol/vol) and incorporation of radioactivity into cholesteryl esters was determined as described above. Protein was determined by the method of Lowry *et al.* (6) with BSA as the standard.

Statistical analysis. The unpaired Student's *t*-test was used to determine significance.

RESULTS AND DISCUSSION

Figure 1 shows the time course of cholesteryl ester formation in Caco-2 cells. Intracellular cholesteryl [^{14}C]-oleate increased with incubation time and reached the maximum at 5 h. When cells were treated with cholesterol or 25-HOC, cholesteryl [^{14}C]oleate formation increased (Fig. 2). 25-HOC was more potent than cholesterol. When 10 μM 25-HOC was present, the formation of cholesteryl esters was twice that of controls.

As shown in Figure 3, cholesteryl ester formation was stimulated by 120%, while incorporation of [^{3}H]leucine into 5% trichloracetic acid-insoluble material decreased when cells were treated with 25-HOC. Upon addition of cycloheximide or actinomycin D to the culture which had received 25-HOC, the incorporation of [^{3}H]leucine into the cells decreased by 87% or 40%, respectively. However, the stimulation of cholesteryl ester formation was not

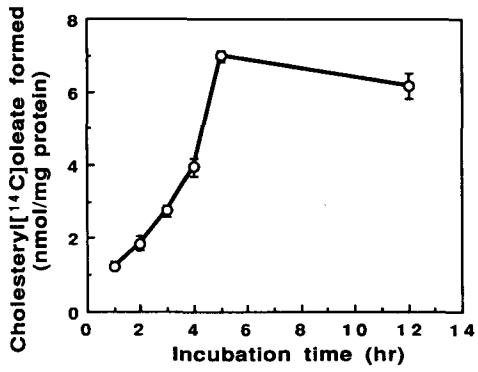

FIG. 1. Intracellular cholesteryl ester formation in Caco-2 as a function of incubation time. Caco-2 cells, grown to confluence in 12-well culture plates, received 1 mL of DMEM containing 100 μM [^{14}C]-oleic acid and 20 μM BSA at 0 h. Lipids were extracted from cells with *n*-hexane/2-propanol and the amount of cholesteryl [^{14}C]oleate was determined after separation on thin-layer plates. The data are the mean and SD from triplicate assays.

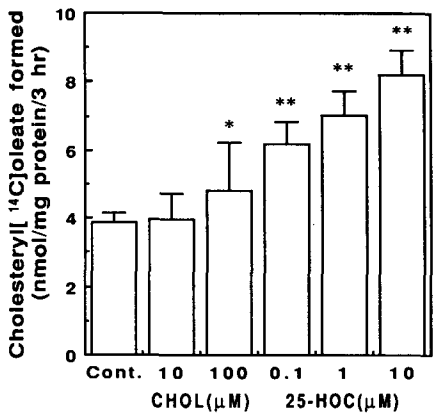

FIG. 2. Effects of exogenous cholesterol (CHOL) and 25-hydroxycholesterol (25-HOC) on cholesteryl ester formation in Caco-2 cells. Cells were treated with the indicated amount of sterols for 2 h. They were then incubated 3 h with [^{14}C]oleic acid and BSA. The data are the mean and SD from triplicate assays, *, $P < 0.05$; **, $P < 0.01$, *vs.* control.

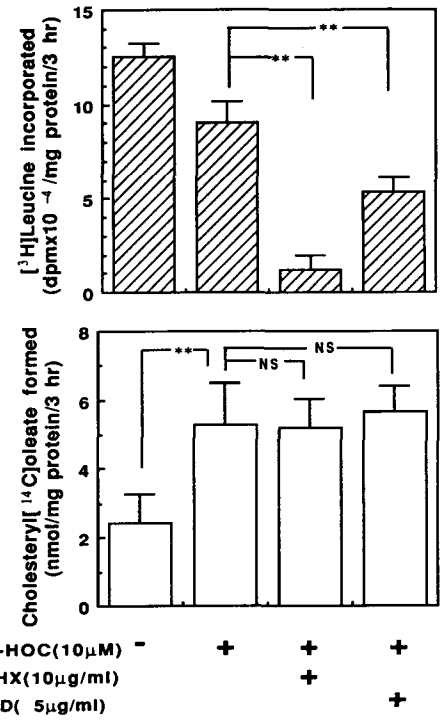

FIG. 3. Effects of cycloheximide (CHX) and actinomycin D (AD) on the stimulation of cholesteryl ester formation by 25-hydroxycholesterol (25-HOC) in Caco-2. Cells were treated with 10 μM 25-HOC plus cycloheximide (10 μg/mL) or actinomycin D (5 μg/mL) for 2 h. They were then incubated for 3 h with [^{14}C]oleic acid, BSA and [^{3}H]leucine. After lipids were extracted, cells were solubilized in 1 mL of 1 N NaOH. [^{3}H]Leucine incorporated in the cells was determined by counting the radioactivity of the solution after neutralization. The data are the mean and SD from triplicate assays, **, $P < 0.01$; NS, not significant.

affected by these inhibitors. This shows that protein synthesis is not required for stimulation of cholesteryl ester formation in Caco-2 cells.

Figure 4 shows the effect of staurosporine, an inhibitor of protein kinases, on the stimulation of cholesteryl ester formation by 25-HOC. The formation of cholesteryl esters was suppressed by staurosporine only when 25-HOC was present. This suggests that protein phosphorylation is involved in the stimulation of cholesteryl ester formation.

The specific activity of ACAT in microsomes prepared from cells treated with 10 μM 25-HOC was 202 $\pm$ 21 pmol cholesteryl [^{14}C]oleate/mg protein/min (n = 5), whereas ACAT activity of controls was 105 $\pm$ 30 pmol cholesteryl [^{14}C]oleate/mg protein/min (n = 5). The difference is statistically significant ($P < 0.01$). ACAT activity in control microsomes was 92 $\pm$ 4 pmol cholesteryl [^{14}C]oleate/mg protein/min (n = 3) and 90 $\pm$ 14 pmol cholesteryl [^{14}C]oleate/mg protein/min (n = 3) when incubated with or without 10 μM 25-HOC for 1 h, respectively. This suggests that ACAT activity would not be enhanced by microsomal enzymes during the incubation with 25-HOC.

It has been reported that ACAT activity in rat liver and intestine can be modulated by phosphorylation/dephosphorylation (7,8). Gavey *et al.* (8) have demonstrated that ACAT can be inactivated by incubation without phosphatase inhibitors and reactivated by incubation with a

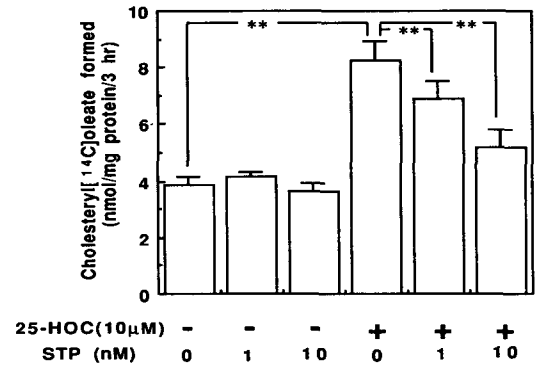

FIG. 4. Effects of staurosporine (STP) on the stimulation of cholesteryl ester formation by 25-hydroxycholesterol (25-HOC). Cells were treated with 10 μM 25-HOC and the indicated amount of staurosporine. The data are the mean and SD from triplicate assays, **, $P < 0.01$.

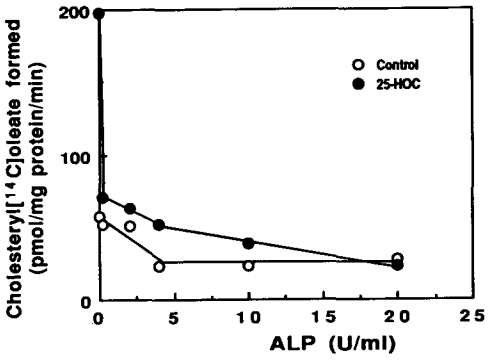

FIG. 6. Inactivation of microsomal ACAT by incubation with exogenous phosphatase. Microsomes (40 μg protein) were preincubated at 37°C for 30 min with the indicated amount of *E. coli* alkaline phosphatase in 154 mM potassium phosphate buffer (pH 7.4), in a total volume of 200 μL, prior to ACAT assay. The data are the average of duplicate determinations.

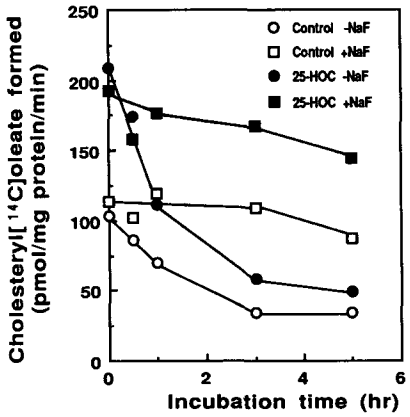

FIG. 5. Inactivation of microsomal ACAT by incubation without sodium fluoride. Microsomes (40 μg protein) were preincubated with or without 100 mM NaF in 100 mM imidazole buffer (pH 7.4) at 37°C for 5 min, in a total volume of 200 μL. Then the ACAT assay was carried out by incubation upon addition of 20 μM [^{14}C]oleoyl-CoA and 20 μM BSA in 154 mM potassium phosphate buffer (pH 7.4) at 37°C for 10 min, in a total volume of 500 μL. The reaction was stopped by addition of 5 mL of chloroform/methanol (2:1, vol/vol). Lipids were extracted and separated on thin-layer plates. The data are the average of duplicate determinations.

partially purified kinase. Figure 5 shows the stability of ACAT activity in Caco-2 microsomes when incubated with or without 100 mM sodium fluoride, a phosphatase inhibitor, at 37°C. ACAT activity in microsomes decreased in the absence of NaF, with the decrease in the microsomes from cells treated with 25-HOC being more notable. The decrease in microsomal ACAT activity was suppressed by NaF. ACAT activity in microsomes was also sensitive to *E. coli* alkaline phosphatase as shown in Figure 6. The activity in microsomes from cells treated with 25-HOC fell to almost the same value as that in microsomes from control cells, when incubated with 20 units/mL of phosphatase for 30 min. These results indicate that ACAT in the cells treated with 25-HOC is more sensitive to endogenous and exogenous phosphatases than ACAT in control cells. This and the data from Figure 3, suggest that formation of cholesteryl esters in Caco-2 cells may be stimulated by 25-HOC through either stimulation of phosphorylation or suppression of dephosphorylation of ACAT; however, phosphorylation of the protein remains to be demonstrated.

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Occurrence of a Novel Sterol, 24,25-Methylenecholest-5-en-3β-ol, in *Mortierella alpina* 1S-4

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24,25-Methylenecholest-5-en-3β-ol, which has not been reported previously to exist in nature, was isolated from mycelia of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4. Desmosterol, ergosta-5,24(25)-dien-3β-ol and ergosta-5,25-dien-3β-ol were also found in the fungus, but ergosterol and cholesterol were not detected.
***Lipids* 27, 481–483 (1992).**

Sterols with a cyclopropane (or cyclopropene) ring have been reported to exist in sponges or coelenterates, *i.e.*, gorgosterol (5) (1–3), 23-demethylgorgosterol (6) (4), calysterol (7) (5), papakusterol (8) (6), and petrosterol (9) (7,8). They are all contained in marine lower organisms. On the other hand, many of 24-methylsterols are found in plants and fungi (9).

During the course of our studies on the lipid metabolism in an arachidonic acid-producing zygomycetous fungus, *Mortierella alpina* 1S-4, (10–20), we found that the fungus produces a novel sterol with a cyclopropane ring, 24,25-methylenecholest-5-en-3β-ol (4) besides desmosterol (1) and 24-methylsterols, *i.e.*, ergosta-5,25-dien-3β-ol (2) and ergosta-5,24(25)-dien-3β-ol (3). To our knowledge, the occurrence of such a cyclopropane sterol in nature has not been reported. We reported here the isolation and identification of sterol 4 from the fungal mycelia.

MATERIALS AND METHODS

Chemicals. Desmosterol was purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol and ergosterol were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals used in this work were of analytical grade and commercially available.

Microorganism, medium and cultivation. Mortierella alpina 1S-4 (AKU 3998; Faculty of Agriculture, Kyoto University, Kyoto, Japan) (10,17) was used. The fungus was cultivated in a medium (20 mL, pH 6.0) containing 2% glucose and 1% yeast extract in a 100-mL flask for 6–8 days at 28°C with reciprocal shaking (120 strokes/min).

Extraction and analysis of sterols. Fungal mycelia obtained on suction filtration were washed with water and then dried at 100°C for 2 h. Fifty mg of dried mycelia was incubated with 5 mL of 33% KOH/methanol (1:4, vol/vol) at 70°C for 1 h. After extraction with 10 mL of *n*-hexane, followed by evaporation, the unsaponifiable fraction was analyzed by gas-liquid chromatography (GLC) or high-performance liquid chromatography (HPLC). The analytical conditions were as follows. GLC: apparatus, GC-7A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector; column, ULBON HR-1 (0.25 mm × 25 m) (Shinwakakou, Kyoto, Japan); column temperature, 280°C; injection port temperature, 300°C; carrier gas, He (2 mL/min); make-up gas, N_2 (60 mL/min); split ratio, 30:1. HPLC: pump, LC-6A (Shimadzu); column, A-302 (S-5 120A ODS) (Yamamura Chemical Lab., Kyoto, Japan); detector, SPD-6A (Shimadzu); wave length, 210 nm; mobile phase, acetonitrile/methanol/water (19:19:2, vol/vol/vol); flow rate, 1.5 mL/min; column temperature, 35°C. Cholesterol was used as an internal standard for both GLC and HPLC analyses.

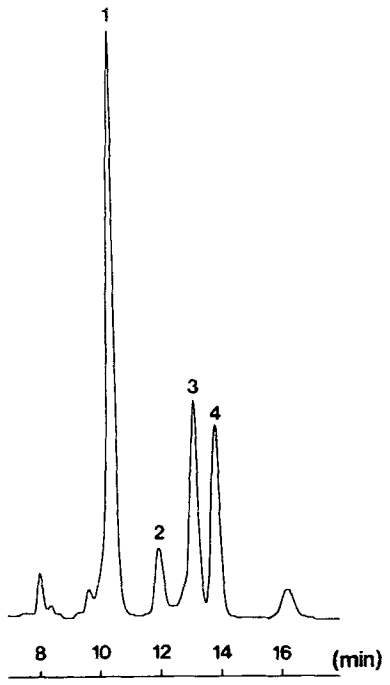

FIG. 1. HPLC chromatogram of the unsaponifiable fraction of mycelia of *Mortierella alpina* 1S-4.

Determination of the structure of the isolated sterols. Each mycelial sterol was isolated by HPLC using the conditions described above except for the following: column, Cosmosil 5C18 (10 × 250 mm, Nakalai Tesque, Kyoto, Japan); mobile phase, acetonitrile/methanol (1:1, vol/vol); and flow rate, 3.5 mL/min. Mass spectra of the isolated sterols were measured with Hitachi (Tokyo, Japan) MK-80 instrument (ionization potential, 70 eV). ^1H Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a Nicolet (Fermont, CA) NT-360 (360 MHz) with tetramethylsilane as an internal standard.

RESULTS AND DISCUSSION

The nonsaponifiable fraction obtained from mycelia of *Mortierella alpina* 1S-4 was analyzed by HPLC and GLC. Four major peaks were detected by HPLC analysis (Fig. 1). They all were different from the common fungal sterols,

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[1]On leave from Suntory Ltd.

Abbreviations: EIM, electron impact mass spectrometry; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; RRT, relative retention time(s).

TABLE 1

Analytical Data for Sterols of *Mortierella alpina* 1S-4

Analysis	Values for sterols (1)	(2)	(3)	(4)
^{1}H NMR				
H-3	3.53(*m*,1H)	3.53(*m*,1H)	3.53(*m*,1H)	3.53(*m*,1H)
H-6	5.37(*m*,1H)	5.37(*m*,1H)	5.37(*m*,1H)	5.37(*m*,1H)
H-18	0.68(*s*,3H)	0.68(*s*,3H)	0.68(*s*,3H)	0.68(*s*,3H)
H-19	1.01(*s*,3H)	1.01(*s*,3H)	1.01(*s*,3H)	1.01(*s*,3H)
H-21	0.91(*d*,3H)	0.91(*d*,3H)	0.91(*d*,3H)	0.91(*d*,3H)
H-24	5.09(*m*,1H)	n.d.[a]	—[b]	−0.16(*m*,1H)
H-26	1.60(*s*,3H)	4.65(*s*,2H)	1.61(*s*,3H)	1.01(*s*,3H)
H-27	1.68(*s*,3H)	1.62(*s*,3H)	1.61(*s*,3H)	1.01(*s*,3H)
H-28	—[b]	0.98(*d*,3H)	1.61(*s*,3H)	0.37(*m*,2H)
EIMS				
M^+	384, 22%	398, 69%	398, 10%	398, 25%
M^+−Me	369, 28%	383, 17%	383, 6%	383, 13%
M^+-H_2O	366, 5%	380, 10%	380, 0%	380, 5%
M^+-Me-H_2O	351, 12%	365, 14%	365, 4%	365, 14%
M^+-SC^c-2H	271, 87%	271, 40%	271, 21%	271, 95%
base peak	69, 100%	55, 100%	83, 100%	69, 100%
GLC RRT[d]	1.07	1.18	1.34	1.18
HPLC RRT	0.69	0.78	0.85	0.89
Content (mg/g dry mycelia)	3.11	0.56	0.50	1.15

[a]n.d., not determined.

[b]Respective hydrogen lacking.

[c]SC, side chain.

[d]RRT, relative retention time in respect to cholesterol.

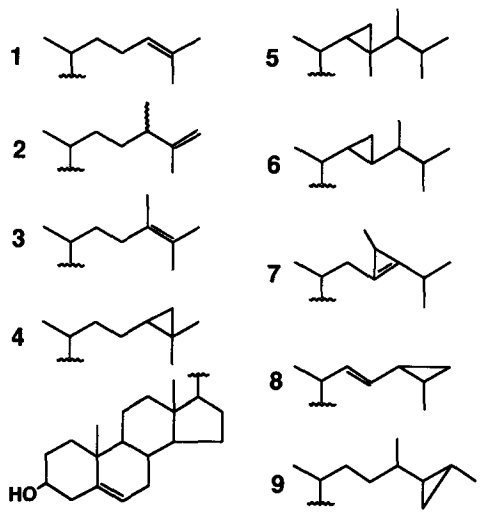

FIG. 2. Structures of sterols from *Mortierella alpina* 1S-4 (1–4) and from sponges or coelenterates (5–9).

ergosterol and cholesterol. Four sterols (1–2 mg each) were isolated by HPLC on a reversed phase column and examined by mass spectrometry (MS) and ^{1}H NMR analyses (Table 1). The ^{1}H NMR spectrum of the C_{27} sterol (1) indicates Δ^5 and Δ^{24} double bonds and is shown to be identical with that of authentic desmosterol (cholesta-5,24-dien-3β-ol) (Fig. 2). Compounds 2 and 3 were determined to be C_{28} sterols with two double bonds, *i.e.*, ergosta-5,25-dien-3β-ol and ergosta-5,24(25)-dien-3β-ol, respectively. The ^{1}H NMR spectrum of compound 4 showed signals at δ −0.16 and 0.37 ppm (Table 1), indicating that this compound contains a cyclopropane ring. The data in Table 1 suggest that compound 4 is 24,25-methylenecholest-5-en-3β-ol; the data are identical with those of the synthetic compound prepared by addition of dichlorocarbene to desmosterol, followed by metal reduction (21,22). This is the first report on the existence of compound 4 in nature and of the occurrence of a sterol with a cyclopropane ring in a fungus. The sterols with a cyclopropane (or cyclopropene) ring reported previously are different from compound 4 in regard to the location of the cyclopropane (or cyclopropene) ring, *i.e.*, 22,23-cyclo- (gorgosterol (5) and 23-demethylgorgosterol (6)), 23,24-cyclo- (calysterol (7)), 24,26-cyclo- (papakusterol (8)) or 26,27-cyclo-structure (petrosterol (9)). The biosynthetic route to 24-methylsterols involving *S*-adenosylmethionine has previously been elucidated (9), but the synthesis of compound 4 is still unclear.

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LETTER TO THE EDITOR

Comments on Essential Fatty Acid Deficient Rats Fed Hydrogenated Oil

Dear Sir:

In their recent paper, Holman *et al.* (1) reported that partially hydrogenated soybean oil (PHSBO) induced the formation of measurable amounts of unusual polyunsaturated fatty acids (PUFA) in rat liver phospholipids in a manner similar to a parallel experiment where fully hydrogenated coconut oil was fed to cause an essential fatty acid deficiency (EFAD). We have reviewed this publication, conferred with the two senior authors (1), and retrieved the compositional data on the particular type of PHSBO which was used in this study that industry had prepared for the Holman group. This letter seeks to clarify and explain the results obtained by Holman *et al.* (1).

First, it must be noted that no data were presented in the paper as to the fatty acid compositions of the fats employed (1). This unfortunately precludes replication of the experiments or direct analysis of the results. However, in private conversations, both H. J. Dutton and R. T. Holman confirmed that their PHSBO treatment group received only the PHSBO supplied by industry. No supplemental essential fatty acid (EFA) was added to this diet. Industry records (Kuss, G., private communication) show that the PHSBO samples used in this study contained a high *trans* isomer level (45–48% of total fatty acids) with essentially no linoleic acid (0–0.8% of total fatty acids). This level of linoleic acid in the diet (est. 0–0.08%) is, at best, well below the 0.6% recommended by the National Research Council (NRC) for EFA adequacy in the rat (2). Thus, although the authors noted (1) "... the PHSO contained sufficient linoleic acid ... to prevent dermatitis of EFAD," the rats in question certainly did not have adequate EFA in their diets based on industry analyses and NRC recommendations. Furthermore, examination of the data in Table 1 (1) reveals that both the EFAD and PHSBO diets produced 20:3/20:4 ratios of 2.8 and 1.3, respectively, in liver phospholipids; these are far in excess of the 0.4 maximum first described by Holman in 1960 (3) as the upper limit of EFA adequacy. Ratios in excess of 0.4 are indicative of EFAD. Based on all of these facts and the similarities of the results obtained in the two experimental diets, we believe the data reported resulted from EFAD in both groups of rats and that a so-called "isomeric" effect with the PHSBO has not been demonstrated.

A similar PHSBO was used by Kummerow *et al.* (4,5) in feeding studies on swine which led to severe aortic atherosclerosis in pigs fed only the PHSBO as their dietary fat. However, later repetition (6,7) of these studies in which adequate EFA was provided revealed no significant atherosclerosis in the swine fed the PHSBO. In addition, Zevenbergen *et al.* (8) conducted a study, not cited by Holman *et al.* (1), which was similar to Holman *et al.*'s (1) study in that rats were fed a PHSBO diet with limited amounts of EFA. Importantly, Zevenbergen *et al.* (8) demonstrated that supplementation of the PHSBO diet with 2 energy percent of linoleic acid prevented undesirable effects on mitochondrial function and tissue lipid polyunsaturated fatty acid composition associated with high amounts of *trans* fatty acids in the diets with limited EFA.

Finally, we believe that Holman *et al.*'s (1) concern that "large-scale hydrogenation of vegetable oils ... (induces) significant partial deficiencies of EFA" is completely unwarranted. The NRC's Food and Nutrition Board (9) has concluded that EFAD is simply not a health problem for the average adult in the U.S. because the "minimally adequate intake of linoleic acid ... (3 to 6 g/day) ... is more than met by diets in the United States. ..."

In spite of the great significance for humans that was attributed to these data (1), we believe that these conclusions are without basis in fact and that repetition of the experiments with added EFA will reveal that there is no significant "isomeric" effect. Further, as we (10,11) and the NRC (12) have emphasized, the findings of the Federation of American Societies for Experimental Biology (FASEB) committee (13) are still valid, and there is no hazard associated with the typical intake of hydrogenated fats in U.S. diets (11,14) where adequate EFA is present.

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Abbreviations: EFA, essential fatty acid; EFAD, essential fatty acid deficiency; FASEB, Federation of American Societies for Experimental Biology; NRC, National Research Council; PHSBO, partially hydrogenated soybean oil; PUFA, polyunsaturated fatty acid.

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Essential Fatty Acid Deficient Rats Fed Hydrogenated Oil —A Response

Dear Sir:

Our recent report, that ingestion of partially hydrogenated soybean oil (PHSO) is accompanied by unusual isomeric polyunsaturated fatty acids (PUFA) in the livers of rats (1), has prompted T. H. Applewhite and J. E. Hunter to respond in the preceding Letter to the Editor of this journal. They believe that the data we reported "resulted from EFAD in both groups of rats and that a so-called 'isomeric' effect with PHSO has not been demonstrated."

The PHSO furnished to us by industry for these studies contained 45 to 48% *trans* unsaturated acids but a maximum of 0.8% *cis,cis*-linoleic acid by their analyses. Our attempt to measure the linoleic acid content of the 18:2 fraction from the PHSO sample failed because the complexity of the mixture exceeded the limits of our method. Accepting their analysis, 10% of PHSO in the diet could thus have provided no more than 0.08% of linoleic acid in the diet, or approximately 0.16% of calories. This, indeed, was well below the 0.6% of calories recommended by the National Research Council (NRC) committee.

The purpose of our study was to detect, identify and measure the individual 18:2 isomers in liver lipids of rats in two models of essential fatty acid deficiency (EFAD) known or suspected to develop elevated levels of unusual isomers of 18:2, and to compare them with controls receiving adequate linoleic acid. Our results indicate that, indeed, unusual positional isomers of 18:2, 20:2, 20:3 and 20:4 can and do occur in the liver of rats made essential fatty acid (EFA) deficient by low EFA and high saturated fat or high levels of unusual isomers of 18:1.

The major difference between the diet groups was that EFAD rats received high levels of 12- to 16-carbon saturated fatty acids, whereas the PHSO group received high levels of positionally isomeric *cis* and *trans* 18:1 fatty acids. The 45–48% of *trans* isomers measured by the donor of the sample must have been accompanied by 22.5 to 24% of *cis*-positional isomers as well, for hydrogenation yields an equilibrium mixture of about 2 parts *trans* to 1 part *cis*. Total *cis*- and *trans*-18:1 positional isomers comprised two-thirds of the dietary fatty acids, and both groups of isomers are substrates for liver microsomal desaturases (2).

The two models *did not* induce the same pattern of positional isomers of 18:2 and 20:2 in liver phospholipids despite their common feature of low or deficient levels of linoleic acid. The saturated fat diet used to induce EFA deficiency produced several endogenously synthesizable 18:2 and 20:2 isomers which were identified. The PHSO diet, containing 6.75 to 7.2% of isomeric 18:1 acids, induced some of the same 18:2 and 20:2 isomers, but *additional isomers also appeared* which were not found in simple EFA deficiency. Isomeric 18:1, and perhaps isomeric 18:2, provided by PHSO are the most plausible precursors of some of the isomers of 18:2 and 20:2 peculiar to PHSO-fed rat liver (2).

Both saturated fatty acids (EFAD diet) and positionally isomeric 18:1 (PHSO diet) suppressed the content of essential arachidonic acid in the liver phospholipids (PL), indicating impairment of EFA metabolism, as had been reported previously for rats fed low EFA and high isomeric 18:1 (3,4).

Positionally isomeric 18:1 present in dietary, partially hydrogenated fat interfere with the normal metabolism of polyunsaturated fatty acids (PUFA) by providing alternative substrates for desaturation and elongation, the products of which compete with naturally-occurring PUFA at several steps in the metabolic cascade. This effect becomes pronounced when the diet contains an inadequate level of linoleic acid. We are fully aware that the products of positional isomers of 18:1, as well as the products from oleic acid, are suppressed by increase of dietary linoleic acid, a preferred substrate (5). The proportions of competing substrates and their affinities for the enzymes govern the proportions of the products, and, in our opinion, even when linoleic acid is "adequate" by committee standards, unusual isomers of PUFA can be produced from unusual isomers of 18:1. Our point is that unusual isomers of PUFA become measureable when EFA intake is low.

In quoting our statement that "Large-scale hydrogenation of vegetable oils . . . (induces) significant partial deficiencies of EFA," Applewhite and Hunter did not include the main point of our concern: Linolenic acid, now known to be an essential fatty acid, is abundant in natural soybean oil but is severely diminished in hydrogenated products. The remainder of our statement was: "It would seem wise to preserve the essential nutrients and to avoid producing inhibitors of their metabolism by hydrogenation. Evidence is growing for the essentiality of ω3 PUFA and the occurrence of deficiencies of ω3 acids in humans under stress conditions (ref. 6 here). It would therefore, be wise economy to use oils containing linolenic acid directly as foods and to avoid their hydrogenation."

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Abbreviations: EFA, essential fatty acids; EFAD, essential fatty acid deficiency; NRC, National Research Council; PHSO, partially hydrogenated soybean oil; PUFA, polyunsaturated fatty acids.

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ARTICLE

Linoleic Acid Metabolism in the Red Alga *Lithothamnion corallioides*: Biosynthesis of 11*(R)*-Hydroxy-9*(Z)*,12*(Z)*-octadecadienoic Acid

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Incubation of [1-^{14}C]linoleic acid with an enzyme preparation obtained from the red alga *Lithothamnion corallioides* CROUAN resulted in the formation of 11-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid as well as smaller amounts of 9-hydroxy-10*(E)*,12*(Z)*-octadecadienoic acid, 13-hydroxy-9*(Z)*,11*(E)*-octadecadienoic acid and 11-keto-9*(Z)*,12*(Z)*-octadecadienoic acid. Steric analysis showed that the 11-hydroxyoctadecadienoic acid had the *(R)* configuration. The 9- and 13-hydroxyoctadecadienoic acids were not optically pure, but were due to mixtures of 75% *(R)* and 25% *(S)* enantiomers (9-hydroxyoctadecadienoate), and 24% *(R)* and 76% *(S)* enantiomers (13-hydroxyoctadecadienoate). 11-Hydroxyoctadecadienoic acid was unstable at acidic pH. In acidified water, equal parts of 9*(R,S)*-hydroxy-10*(E)*,12*(Z)*-octadecadienoate and 13*(R,S)*-hydroxy-9*(Z)*,11*(E)*-octadecadienoate, plus smaller amounts of the corresponding *(E),(E)* isomers were produced. In aprotic solvents, acid treatment resulted in dehydration and in the formation of equal amounts of 8,10,12- and 9,11,13-octadecatrienoates. The enzymatic conversion of linoleic acid into the hydroxyoctadecadienoic acids and the ketooctadecadienoic acid was oxygen-dependent; however, inhibitor experiments indicated that neither lipoxygenase nor cytochrome P-450 were involved in the conversion. This conclusion was supported by experiments with $^{18}O_2$ and $H_2{}^{18}O$, which demonstrated that the hydroxyl oxygen of the hydroxyoctadecadienoic acids and the keto oxygen of the 11-ketooctadecadienoic acid were derived from water and not from molecular oxygen.

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Studies carried out during the last years have documented the importance of lipoxygenases in the metabolism of polyunsaturated fatty acids in marine algae (for a review, see ref. 2). The majority of oxylipins thus produced are formed by sequences that involve arachidonic acid 12-lipoxygenase in the initial step. An example of this is the conversion of arachidonic acid into 12*(R)*,13*(S)*-dihydroxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid (12*(R)*,13*(S)*-diHETE) (3), a transformation that occurs by initial lipoxygenase-catalyzed oxygenation of arachidonic acid into 12*(S)*-hydroperoxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid (12*(S)*-HPETE), followed by hydroperoxide isomerase-catalyzed conversion of the hydroperoxide into the diol fatty acid (1).

Guerriero *et al.* (4) recently isolated the ethyl esters of an array of oxylipins from the calcareous red algae *Lithothamnion corallioides* and *Lithothamnion calcareum.* Apart from the previously known esters of 5-, 11-, 12- and 15-hydroxyeicosatetraenoic acids (5-, 11-, 12- and 15-HETE), three new esters oxygenated at C-13 were obtained, *i.e.,* ethyl 13-hydroxy-5*(Z)*,8*(Z)*,11*(Z)*,14*(Z)*-eicosatetraenoate, ethyl 13-hydroxy-5*(Z)*,8*(Z)*,11*(Z)*,14*(Z)*,17*(Z)*-eicosapentaenoate and ethyl 8-hydroxy-13-keto-5*(Z)*,9*(E)*,11*(E)*,14*(E)*-eicosatetraenoate. The unusual structures of the three latter compounds prompted an investigation of the metabolism of ^{14}C-labeled polyunsaturated fatty acids in *Lithothamnion.* The present work is concerned with transformations of [^{14}C]linoleic acid in the presence of an enzyme preparation of *Lithothamnion corallioides* and with the reaction mechanism.

EXPERIMENTAL PROCEDURES

Linoleic acid was purchased from NuChek Prep, Inc. (Elysian, MN). [1-^{14}C]Linoleic acid was obtained from Amersham Laboratories (Amersham, U.K.) and added to the unlabeled acid to provide a specimen having a specific radioactivity of 5.3 kBq/μmol. 9*(R,S)*-HOD, 9*(R,S)*-hydroxy-10*(E)*,12*(E)*-octadecadienoic acid, 13*(R,S)*-HOD and 13*(R,S)*-hydroxy-9*(E)*,11*(E)*-octadecadienoic acid were prepared by treatment of the corresponding hydroperoxides (5) with sodium borohydride. Methyl 11-hydroxy-12-octadecenoate was obtained by deoxygenation of methyl 11-hydroxy-12,13-epoxyoctadecanoate (Hamberg, M., unpublished data). [9,10,12,13-$^2H_4$]Linoleic acid was prepared by partial deuteriation of 9,12-octadecadiynoic acid as described previously (6). The isotopic composition of the sample was 93% tetradeuteriated and 7% trideuteriated molecules. $^{18}O_2$ (97–98%) was obtained from Cambridge Isotope Laboratories (Woburn, MA) and $H_2{}^{18}O$ (98.7 atom%) was purchased from Isotec, Inc. (Miamisburg, OH).

Enzyme preparation. The red alga *Lithothamnion corallioides* CROUAN was collected at 10–15 m depth in a semi-exposed sound on the Southern coast of Norway in June, 1991, and kept at −77°C. Batches of about 90 g of frozen algae were crushed and ground in a mortar with liquid nitrogen. The coarse powder (85 g) was added to 0.09 M potassium phosphate buffer, pH 7.4 (85 mL), and homogenized at 0°C with an Ultra-Turrax (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 9,300 × *g* for 15 min and the resulting supernatant was further centrifuged at 105,000 × *g* for 60 min.

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Abbreviations: ETYA, 5,8,11,14-eicosatetraynoic acid; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; 12*(R)*,13*(S)*-diHETE, 12*(R)*,13*(S)*-dihydroxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6*(E)*,8*(Z)*,11*(Z)*,14*(Z)*-eicosatetraenoic acid; 11-HETE, 11-hydroxy-5*(Z)*,8*(Z)*,12*(E)*,14*(Z)*-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5*(Z)*,8*(Z)*,11*(Z)*,13*(E)*-eicosatetraenoic acid; 9-HOD, 9-hydroxy-10*(E)*,12*(Z)*-octadecadienoic acid; 13-HOD, 13-hydroxy-9*(Z)*,11*(E)*-octadecadienoic acid; 12*(S)*-HPETE, 12*(S)*-hydroperoxy-5*(Z)*,8*(Z)*,10*(E)*,14*(Z)*-eicosatetraenoic acid; MC, (−)-menthoxycarbonyl; Me_3Si, trimethylsilyl; NDGA, nordihydroguaiaretic acid; TLC, thin-layer chromatography. The term "oxylipin" was introduced recently (ref. 1) as an encompassing term for oxygenated compounds which are formed from fatty acids by reaction(s) involving at least one step of mono- or dioxygenase-catalyzed oxygenation.

Ammonium sulfate fractionation carried out with the high speed supernatant provided precipitates obtained at 0-30%, 30-55% and 55-80% saturation. The 30-55% precipitate, which contained more than 90% of the linoleic acid oxidizing activity, was dissolved in potassium phosphate buffer and used as the enzyme source. The protein concentration (7) was 2 mg/mL. If desired, the 30-55% ammonium sulfate precipitate could be stored at −30°C for several weeks without loss of activity.

Incubations and treatments. [1-^{14}C]Linoleic acid (300 μM) was stirred with enzyme preparation at 22°C for the times indicated. Five volumes of methanol were added and the mixture diluted with water, acidified to pH 4, and rapidly extracted with two portions of diethyl ether. The combined ether phases were washed until neutral reaction and dried over magnesium sulfate. The product obtained after evaporation of the ether was esterified by treatment with diazomethane and subjected to thin-layer radiochromatography. Recovery of radioactivity from added labeled substrate was 90-95%.

Chemical methods. Hydrogenation was carried out with palladium on calcium carbonate as the catalyst (8). Preparation of (−)-menthoxycarbonyl (MC) derivatives and procedures for oxidative ozonolysis and steric analysis of MC derivatives by gas-liquid chromatography (GLC) were performed as described (9,10).

Chromatographic and instrumental methods. Thin-layer chromatography (TLC) was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck (Darmstadt, Germany). The solvent systems consisted of mixtures of ethyl acetate/hexane in the proportions indicated. Thin-layer argentation chromatography was performed with plates coated with Silica gel G/$AgNO_3$ (9:1, w/w) and a solvent system consisting of ethyl acetate/hexane (25:75, vol/vol). Radioactivity on TLC plates was determined with a Berthold Dünnschichtscanner II (Wildbad, Germany) interfaced with a Macintosh SE/30 PC. GLC was carried out with a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Gas chromatography/mass spectrometry (GC/MS) was performed with a Hewlett-Packard Model 5970B mass selective detector connected to a Hewlett-Packard Model 5890 gas chromatograph. Ultraviolet spectroscopy was carried out with a Hewlett-Packard Model 8450A diode array spectrophotometer, and infrared spectra were obtained with a Perkin-Elmer (Überlingen, Germany) model 257 infrared spectrrophotometer. Radioactivity was measured with a Packard (Downers Grove, IL) Tri-Carb Model 4450 liquid scintillation counter.

RESULTS

Isolation of products formed from linoleic acid. Figure 1A shows a thin-layer radiochromatogram of the esterified product obtained following incubation of 300 μM [1-^{14}C]-linoleic acid with the enzyme preparation at 22°C for 20 min. The reaction product consisted of three compounds (Compounds A_1, A_2 and A_3) having a polarity typical for hydroxyoctadecadienoates, as well as a fourth compound (Compound B; $R_f = 0.52$), which migrated like a ketooctadecadienoate. Compound B was directly obtained in a purity which was satisfactory for structural analysis. In order to obtain Compounds A_1-A_3 in pure form, a broad

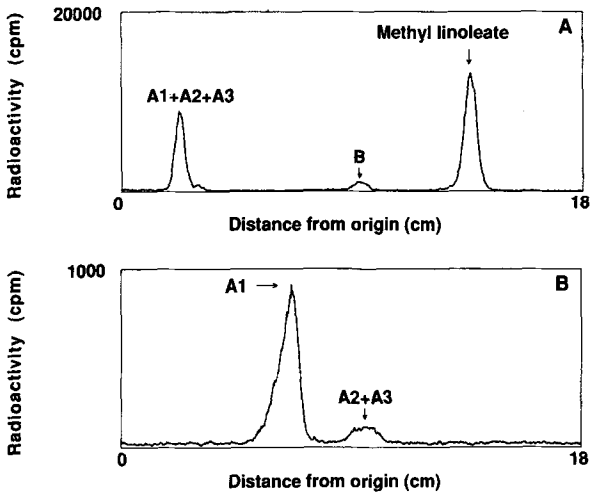

FIG. 1. A: Thin-layer radiochromatogram of esterified product obtained following incubation of [1-^{14}C]linoleic acid (300 μM) with enzyme preparation (2 mL) at 22°C for 20 min. Solvent system, ethyl acetate/hexane (1:9, vol/vol). B: Thin-layer argentation radiochromatogram of material present in zone of compounds $A_1 + A_2 + A_3$ in A. Solvent system, ethyl acetate/hexane (25:75, vol/vol).

zone of silica gel containing these compounds was scraped off and the material subjected to thin-layer argentation chromatography. As seen in Figure 1B, this resulted in separation of a major hydroxyoctadecadienoate (Compound A_1; $R_f = 0.37$) from two minor hydroxyoctadecadienoates (Compounds A_2 and A_3). The two latter compounds were separated on plain silica gel using a solvent system of ethyl acetate/hexane (2:8, vol/vol; Compound A_2, $R_f = 0.39$; Compound A_3, $R_f = 0.44$).

Structure of Compound A_1. Compound A_1 accounted for 71% of the reaction product formed from linoleic acid. The UV spectrum of Compound A_1 did not show any specific absorption band in the region 200-320 nm, demonstrating the absence of a conjugated diene structure. Infrared spectroscopy showed absorption bands at *inter alia* 3350-3620 cm^{-1} (hydroxyl) and 1735 cm^{-1} (ester carbonyl). No band was observed in the region 950-1000 cm^{-1}, thus excluding the presence of *(E)* double bond(s). Analysis of the Me_3Si derivative of Compound A_1 by GC/MS showed a single peak having a C-value of 19.41. The mass spectrum (Fig. 2) showed prominent ions at *inter alia m/e* 382 (M, 38%), 311 [M − 71; loss of $\cdot(CH_2)_4$-CH_3, 32], and 225 ([CH=CH-CH($OSiMe_3$)-CH=CH-$(CH_2)_4$-CH_3]$^+$, 44). The spectrum was similar to those of the Me_3Si derivatives of the methyl esters of 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HOD). Catalytic hydrogenation performed on Compound A_1 provided methyl 11-hydroxy-stearate as judged by GC/MS analysis of the Me_3Si derivative. Prominent ions appeared at *m/e* 371 (M − 15; loss of $\cdot CH_3$, 4%), 339 [M − (15 + 32), 19], 287 [Me_3SiO^+=CH-$(CH_2)_9$-$COOCH_3$, 100], and 201 [Me_3SiO^+=CH-$(CH_2)_6$-CH_3, 71].

The data mentioned indicated that Compound A_1 was a methyl octadecadienoate having a hydroxyl group at C-11. In order to determine the absolute configuration of C-11 and the positions of the double bonds, a sample of Compound A_1 (250 μg) was stirred with palladium on calcium carbonate (5 mg) in ethyl acetate (3 mL) under

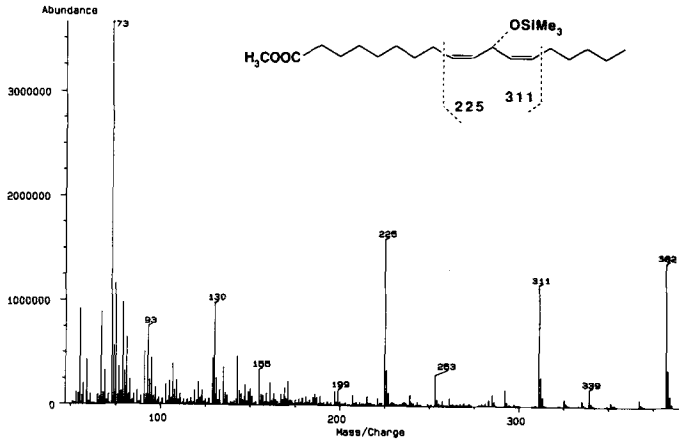

FIG. 2. Mass spectrum of the Me_3Si derivative of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate (Compound A_1).

hydrogen gas for 3 min. The partially hydrogenated product obtained was a mixture of 11-hydroxystearate, two isomeric 11-hydroxyoctadecenoates, and unreacted methyl 11-hydroxyoctadecadienoate as judged by GC/MS analysis. Oxidative ozonolysis performed on the MC derivatives yielded methyl hydrogen azelate (from methyl 11-hydroxy-9,12-octadecadienoate and methyl 11-hydroxy-9-octadecenoate), the MC derivative of 2*(R)*-hydroxynonanoic acid (from methyl 11-hydroxy-9-octadecenoate), as well as the MC derivative of methyl hydrogen 2*(S)*-hydroxy-1,12-dodecanedioate (from methyl 11-hydroxy-12-octadecenoate). On the basis of the results obtained, Compound A_1 was assigned the structure of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate.

Structures of Compounds A_2 and A_3. Compounds A_2 and A_3 each accounted for 5% of the product formed from linoleic acid. The UV spectra of the two compounds showed a strong absorption band at 234 nm (solvent, methanol; $\varepsilon = 26{,}000$), indicating the presence of a conjugated diene structure. The C-value of the Me_3Si derivatives found on GLC analysis of Compounds A_2 and A_3 was 19.81 [references, Me_3Si derivatives of the methyl esters of 9-HOD (C-19.81), 13-HOD (C-19.81), 9-hydroxy-10*(E)*,12*(E)*-octadecadienoic acid (C-20.20), and of 13-hydroxy-9*(E)*,11*(E)*-octadecadienoic acid (C-20.36)]. The mass spectra of the Me_3Si derivative of Compounds A_2 and A_3 were identical with those of the corresponding derivatives of the methyl esters of 9- and 13-HOD, respectively. Prominent ions were observed at *m/e* 382 (M), 311 [M − 71; loss of $\cdot(CH_2)_4$-CH_3] and 225 {due to the ion Me_3SiO^+=CH-$(CH{=}CH)_2$-$(CH_2)_4$-CH_3 in the spectrum of the derivative of 9-HOD, due to the ion [$(CH{=}CH)_2$-$CH(OSiMe_3)$-$(CH_2)_4$-CH_3]$^+$ in the spectrum of the derivative of 13-HOD}. The fragmentation patterns of the derivatives of 9- and 13-HOD were similar; however, the intensity of the *m/e* 225 ion was greater than that of the *m/e* 311 ion in the spectrum of the derivative of 9-HOD, whereas the reverse was true in the spectrum of the derivative of 13-HOD. Catalytic hydrogenation of Compounds A_2 and A_3 produced methyl 9- and 13-hydroxystearates, respectively, as demonstrated by GC/MS. Oxidative ozonolysis performed on the MC derivative of Compound A_2 yielded the MC derivative of methyl hydrogen 2-hydroxysebacate [*(R)/(S)*, 75:25], while the same treatment of the MC derivative of Compound A_3 afforded methyl hydrogen azelate plus the MC derivative of 2-hydroxyheptanoic acid [*(R),(S)*, 24:76]. Thus, Compound A_2 was methyl 9-hydroxy-10*(E)*,12*(Z)*-octadecadienoate [75% of the *(R)* enantiomer], whereas Compound A_3 was identical to methyl 13-hydroxy-9*(Z)*,11*(E)*-octadecadienoate [76% of the *(S)* enantiomer].

Structure of Compound B. Compound B accounted for 13% of the reaction product formed from linoleic acid. The UV spectrum showed a strong absorption band at 255 nm (solvent, methanol; $\varepsilon = 17{,}000$). Mass spectrometric analysis of Compound B showed a molecular ion at *m/e* 308 (6%), as well as high intensity ions at *m/e* 277 (M − 31; loss of $\cdot OCH_3$, 6), 211 [M − 97; loss of $\cdot$CH=CH-$(CH_2)_4$-CH_3, 16], 183 [M − 125; loss of $\cdot$CO-CH=CH-$(CH_2)_4$-CH_3, 16], 151 {[CH=CH-CO-CH=CH-$(CH_2)_4$-CH_3]$^+$, 42}, and 125 [O^+≡C-CH=CH-$(CH_2)_4$-CH_3, 36]. Catalytic hydrogenation of Compound B yielded methyl 11-ketostearate as judged by GC/MS. The data mentioned indicated that Compound B was a methyl 11-ketooctadecadienoate. The ultraviolet spectroscopic data further suggested that the two double bonds were not present as a conjugated diene, but were part of a 3-keto-1,4-pentadiene structure.

Proof for the location and geometry of the double bonds was obtained by an experiment in which the products formed from Compound B upon sodium borohydride reduction were analyzed. Thus, Compound B (240 μg) was treated with $NaBH_4$ (30 mg) in methanol (3 mL) at 22°C for 30 min. Analysis by TLC showed two bands of similar intensity. The more polar material was identified as methyl 11-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate by GC/MS using the authentic compound as reference. The less polar material was due to a 1:1 mixture of methyl 11-hydroxy-9-octadecenoate (C-value of Me_3Si derivative, 19.54; prominent ions at *m/e* 369 (M − 15; loss of $\cdot CH_3$, 1%), 337 [M − (15 + 32), 4] and 285 [Me_3SiO^+=CH-CH=CH-$(CH_2)_7$-$COOCH_3$), 100]) and methyl 11-hydroxy-12-octadecenoate (C-value of Me_3Si derivative, 19.65; prominent ions at *m/e* 369 (M − 15; loss of $\cdot CH_3$, 2%), 337 [M − (15 + 32), 5], and 199 [Me_3SiO^+=CH-CH=CH-$(CH_2)_4$-CH_3, 100; mass spectrum identical with that of the Me_3Si derivative of the authentic compound]. The positions of the hydroxyl group and of the double bond in the two hydroxyoctadecenoates were established by oxidative ozonolysis performed on the MC derivatives. This treatment afforded methyl hydrogen azelate and the MC derivative of 2*(R,S)*-hydroxynonanoic acid (from the MC derivative of methyl 11-hydroxy-9-octadecenoate) and the MC derivative of methyl hydrogen 2*(R,S)*-hydroxy-1,12-dodecanedioate (from the MC derivative of methyl 11-hydroxy-12-octadecenoate). On the basis of the data presented, Compound B was assigned the structure methyl 11-keto-9*(Z)*,12*(Z)*-octadecadienoate.

Acid-induced conversions of methyl 11(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate.* A solution of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate (250 μg) in dimethoxyethane (5 mL) was treated with water (10 mL) and 2 M HCl (0.1 mL) at 22°C for 15 min. Analysis of the product by TLC and GC/MS demonstrated complete conversion of the methyl 11-hydroxyoctadecadienoate into four isomeric hydroxyoctadecadienoates. These compounds were identified, using the authentic materials as references, as the methyl esters of 9-HOD (43%), 13-HOD (43%),

9-hydroxy-10*(E)*,12*(E)*-octadecadienoic acid (7%) and 13-hydroxy-9*(E)*,11*(E)*-octadecadienoic acid (7%). The four hydroxyoctadecadienoates were racemic as shown by oxidative ozonolysis performed on the MC derivatives, which produced the MC derivatives of methyl hydrogen 2*(R,S)*-hydroxysebacate and 2*(R,S)*-hydroxyheptanoic acid.

In another experiment, methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate (27 μg) in methanol (3 mL) was treated with 2 M HCl (2 μL) at 23°C. This treatment was accompanied by the appearance of a UV absorption band with λ_{max} = 234 nm (ε = 27,000). As shown in Figure 3, appearance of the absorption band was rapid. Disappearance of the 11-hydroxyoctadecadienoate in the acidified methanol solution followed first order kinetics with a half-life time of 32 s. The products formed were analyzed by GC/MS and found to be a 1:1 mixture of 9-methoxy-10,12-octadecadienoate [base peak at *m/e* 167, due to CH_3O^+=CH-(CH=CH)$_2$-(CH$_2$)$_4$-CH$_3$] and 13-methoxy-9,11-octadecadienoate [base peak at *m/e* 253, due to CH_3O^+=CH-(CH=CH)$_2$-(CH$_2$)$_7$-COOCH$_3$]. GC/MS analysis of the hydrogenated derivatives was in full accordance with these structures.

The following experiment was carried out in order to investigate the fate of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate in an acidified aprotic solvent. Methyl 11-hydroxyoctadecadienoate (18 μg) in acetonitrile (3 mL) was treated with 70% (w/w) perchloric acid (5 μL). The UV spectrum recorded after 1 min at 23°C showed absorption bands indicative of a conjugated triene, *i.e.*, at 259 nm, 269 nm (ε = 50,000), and 280 nm [Fig. 4; reported for 9*(Z)*,11*(E)*,13*(E)*-octadecatrienoic acid: bands at 261, 270 and 281 nm; and for 9*(E)*,11*(E)*,13*(E)*-octadecatrienoic acid: bands at 258, 268 and 279 nm (11)]. GC/MS analysis of the material showed two pairs of peaks, *i.e.*, at C-18.99 (21%) and 19.11 (21%), and at C-19.41 (29%) and 19.45 (29%). The mass spectra recorded on these peaks showed an intense molecular ion at *m/e* 292, but were otherwise relatively non-informative. Catalytic hydrogenation performed on the product yielded a single compound, *i.e.*, methyl stearate (*m/e* 298). It was thus clear that the product formed upon acid treatment of methyl 11-hydroxy-

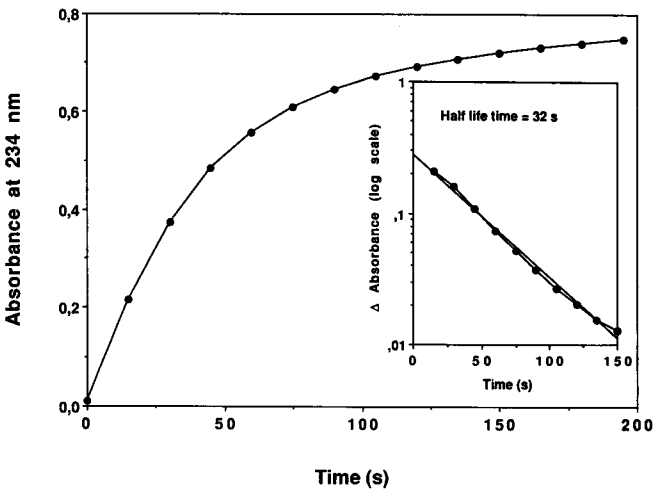

FIG. 3. Appearance of absorbance at 234 nm upon treatment of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate (27 μg) in methanol (3 mL) with 2 μL of 2 M HCl. Inset: Plot of change in absorbance at 234 nm (log scale) *vs.* time.

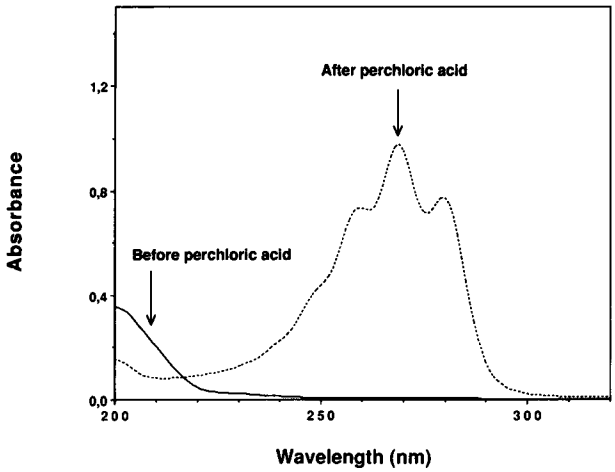

FIG. 4. UV spectra recorded on a solution of 18 μg of methyl 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoate in 3 mL of acetonitrile before (solid line) and 1 min after (dashed line) addition of 5 μL of 70% (w/w) perchloric acid.

octadecadienoate in acetonitrile was due to four isomeric methyl octadecatrienoates. It appeared that the two factors causing separation of the octadecatrienoates upon GLC were different positions of the triene moiety, and geometrical isomerism of the triene. The geometrical configurations of the conjugated trienes were not determined; however, the fact that oxidative ozonolysis performed on the octadecatrienoate mixture yielded equal amounts of methyl hydrogen suberate and methyl hydrogen azelate localized the conjugated triene structures in the carbon chain, and demonstrated that the octadecatrienoates were due to equal amounts of methyl 8,10,12- and 9,11,13-octadecatrienoates.

Incubation of 11-hydroxy- and 11-ketooctadecadienoates. Figure 5A shows the time course of the formation of oxidized products (9-, 11- and 13-hydroxyoctadecadienoic acids plus 11-ketooctadecadienoic acid) from linoleic acid when incubated with the enzyme preparation. As seen, oxidation proceeded linearly in the time interval 0–30 min and then leveled off because of depletion of substrate. No oxidation was observed when heat-inactivated enzyme was used. In order to obtain information of possible interconversions between the 11-oxygenated products, samples of [1-^{14}C]11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid and [1-^{14}C]11-keto-9*(Z)*,12*(Z)*-octadecadienoic acid were prepared by incubation of [1-^{14}C]linoleic acid and isolated as the free acids by silicic acid open column chromatography. The 11-ketooctadecadienoic acid thus obtained was radiochemically pure, whereas the 11-hydroxyoctadecadienoic acid was contaminated with 14% of a mixture of equal parts of 9- and 13-HOD. Re-incubation of [1-^{14}C]11-ketooctadecadienoic acid (100 μM) with the enzyme preparation at 22°C for 20 min did not result in conversion into hydroxyoctadecadienoate(s) or other products. Similar re-incubation of [1-^{14}C]11-hydroxyoctadecadienoic acid resulted in a low yield (5%) of 11-ketooctadecadienoic acid. Slow conversion of 11-hydroxyoctadecadienoic acid into 11-ketooctadecadienoic acid was confirmed by a time-course study (Fig. 5B). Significant formation of 9- and 13-HOD from 11-hydroxyoctadecadienoic acid was not observed.

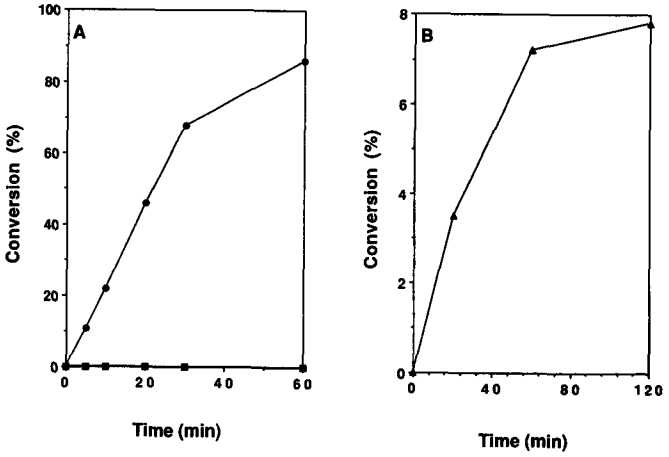

FIG. 5. A: Time course of the oxidation of linoleic acid into 9-, 11- and 13-hydroxyoctadecadienoic acids and 11-ketooctadecadienoic acid. Enzyme preparation (10 mL; -●-●-●-) or heat-inactivated (95°C, 10 min) enzyme preparation (10 mL; -■-■-■-) were stirred with [1-^{14}C]linoleic acid (300 μM) at 22°C and aliquots of 2 mL were removed at times indicated. The percentage conversion of linoleic acid into the three hydroxyoctadecadienoic acids and the 11-keto-octadecadienoic acid was determined by thin-layer radiochromatography. B: Time course of the conversion of 11-hydroxyoctadecadienoic acid into 11-ketooctadecadienoic acid. Enzyme preparation (8 mL) was stirred with [1-^{14}C]11-hydroxyoctadecadienoic acid (100 μM) at 22°C and aliquots of 2 mL were removed at times indicated. The percentage conversion into 11-ketooctadecadienoic acid was determined by thin-layer radiochromatography.

In order to facilitate interpretation of results obtained with linoleic acid incubated in the presence of $^{18}O_2$ and $H_2{}^{18}O$ (see below), re-incubations of 11-hydroxy- and 11-ketooctadecadienoic acids (100 μM) were also carried out with enzyme preparation dissolved in potassium phosphate buffer (1.6 mL) and $H_2{}^{18}O$ (0.4 mL). As seen in Table 1, 11-ketooctadecadienoic acid, either produced from 11-hydroxyoctadecadienoic acid or recovered following incubation of 11-ketooctadecadienoic acid, contained a small fraction of solvent ^{18}O (incorporation, 7-8% of the theoretical). As expected, 11-hydroxyoctadecadienoic acid recovered following incubation of this hydroxy acid did not contain any ^{18}O in the C-11 hydroxyl group.

^{18}O experiments. Linoleic acid (300 μM) was incubated at 22°C for 20 min with the enzyme preparation under an atmosphere of $^{18}O_2$. As seen in Table 2, the 11-hydroxy- and 11-ketooctadecadienoic acids produced were completely devoid of ^{18}O. A small (2.5%) incorporation was observed in 9- and 13-HOD. As a check, following aspiration of the reaction mixture at the end of the incubation period, the incubation vessel was injected with linoleic acid and soybean lipoxygenase. The linoleic acid 13-hydroperoxide thus formed was reduced into 13-HOD. Mass spectrometric analysis of the Me_3Si derivative of the methyl ester of this compound showed satisfactory incorporation of ^{18}O.

In another experiment, linoleic acid was stirred with the enzyme preparation in the presence of $H_2{}^{18}O$. As seen in Table 2, the resulting 11-hydroxy- and 11-ketooctadecadienoates showed virtually complete incorporation of ^{18}O in the hydroxyl and keto groups, respectively. Incorporation of ^{18}O in 9- and 13-HOD was extensive but not complete, *i.e.*, 79-86% of the theoretical.

Incubation of [9,10,12,13-$^2H_4$]linoleic acid. Enzyme preparation (2 mL) was stirred at 22°C for 20 min with 300 μM [9,10,12,13-$^2H_4$]linoleic acid. The hydroxyoctadecadienoates were isolated and converted into the Me_3Si derivatives. Selected monitoring of the ions *m/e* 225, 226, 227, 228 and 229 showed that 11-hydroxyoctadecadienoic acid, as well as 9- and 13-HOD, were due to 93% of tetradeuteriated and 7% trideuteriated molecules. This isotopic composition was the same as that of the incubated deuteriated linoleic acid, thus showing that the hydrogens at carbons 9, 10, 12 and 13 were retained in the conversion of linoleic acid into the three hydroxyoctadecadienoic acids.

Effect of anaerobiosis and enzyme inhibitors. Conversion of linoleic acid into oxidized products was strictly dependent on the presence of oxygen (Table 3). Furthermore, sodium azide in 5 mM concentration inhibited the reaction. The lipoxygenase inhibitor nordihydroguaiaretic acid gave only weak inhibition, and two other well-established lipoxygenase inhibitors, 5,8,11,14-eicosatetraynoic acid and esculetin, were without effect. One cytochrome P-450 inhibitor, metyrapone, lacked inhibitory effect, whereas another one, SKF-525A, gave moderate inhibition when tested in 1 mM concentration. Furthermore,

TABLE 1

Isotope Composition of Compounds Isolated Following Incubation of 11-Hydroxy- and 11-Ketooctadecadienoic Acids with Enzyme Preparation in the Presence of $H_2{}^{18}O$

	Isotope composition (%)[b]			
	11-Hydroxyoctadecadienoic acid		11-Ketooctadecadienoic acid	
Compound incubated[a]	$^{18}O_0$	$^{18}O_1$	$^{18}O_0$	$^{18}O_1$
11-Hydroxyoctadecadienoic acid	100	0 (0)	98.5	1.5 (7)
11-Ketooctadecadienoic acid	—	—	98.4	1.6 (8)

[a] Enzyme preparation (1.6 mL) and $H_2{}^{18}O$ (98.7 atom%, 0.4 mL) were stirred at 22°C for 20 min with 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid (100 μM) or 11-keto-9*(Z)*,12*(Z)*-octadecadienoic acid (100 μM). Five volumes of methanol were added and the products isolated and derivatized as described in the text.

[b] The isotopic compositions were determined by selected monitoring of the following ions: *m/e* 225 and 227 ($[CH=CH-CH(OSiMe_3)-CH=CH-(CH_2)_4-CH_3]^+$, Me_3Si derivative of the methyl ester of 11-hydroxyoctadecadienoic acid, and *m/e* 151 and 153 ($[CH=CH-CO-CH=CH-(CH_2)_4-CH_3]^+$; methyl ester of 11-ketooctadecadienoic acid. The dwell time used for data acquisition was 50 ms. Numbers in parentheses indicate content of ^{18}O relative to the content of ^{18}O in the water of the incubation mixture.

TABLE 2

Isotope Composition of Compounds Isolated Following Incubation of Linoleic Acid with Enzyme Preparation in the Presence of $^{18}O_2$ and $H_2{}^{18}O$

Compound analyzed[a]	Isotope composition (%)			
	Incubation with $^{18}O_2$[b]		Incubation with $H_2{}^{18}O$[c]	
	$^{18}O_0$	$^{18}O_1$	$^{18}O_0$	$^{18}O_1$
11-Hydroxyoctadecadienoic acid	100.0	0	80.1	19.9 (101)
9-HOD	97.5	2.5	83.0	17.0 (86)
13-HOD	97.5	2.5	84.5	15.5 (79)
11-Ketooctadecadienoic acid	100.0	0	80.6	19.4 (98)

[a]The isotopic composition was determined by selected monitoring of the ions *m/e* 225 and 227 (hydroxyoctadecadienoates), and 151 and 153 (11-ketooctadecadienoate).

[b]Enzyme preparation (10 mL) in a sealed vessel was purged with argon and evacuated in five cycles. $^{18}O_2$ (97–98 atom%, 0.1 L) was introduced, and the reaction was started by injection of linoleic acid (300 μM. The mixture was stirred at 22°C for 20 min and then added to five volumes of methanol. Products were isolated and derivatized as described in the text.

[c]Enzyme preparation (4 mL) and $H_2{}^{18}O$ (98.7 atom%, 1 mL) was stirred at 22°C for 20 min with 300 μM linoleic acid. Five volumes of methanol were added and the products isolated and derivatized as described in the text. Numbers in parentheses indicate percentage content of ^{18}O relative to the content of ^{18}O in the water of the incubation mixture.

TABLE 3

Effect of Anaerobiosis and Enzyme Inhibitors on the Oxidation of Linoleic Acid

Condition[a]	Conversion (% of control)[b]
Control	100
Heat-treated enzyme (95°C, 10 min)	0
Anaerobiosis	< 1
Sodium azide (5 mM)	22
SKF-525A (1 mM)	50
Diethyldithiocarbamate (1 mM)	63
NDGA (0.1 mM)	75
2,2′-Dipyridyl (1 mM)	100
Esculetin (0.1 mM)	101
Metyrapone (1 mM)	101
ETYA (0.1 mM)	101

[a]The enzyme preparation was stirred at 22°C for 5 min with inhibitor and then stirred for additional 20 min in the presence of [1-^{14}C]-linoleic acid. Anaerobiosis was accomplished by repeated argon-purging and evacuation of the enzyme preparation contained in a sealed vessel.

[b]The control incubation showed 49% conversion into the three hydroxyoctadecadienoic acids and the ketooctadecadienoic acid.

slight inhibition was noted in the presence of the copper-chelating agent, diethyldithiocarbamate.

DISCUSSION

Several mechanisms exist for introduction of molecular oxygen in the formation of oxylipins. A quantitatively large part of oxylipins is formed by sequences that are initiated by lipoxygenase-catalyzed oxygenation. Compounds formed in this way include leukotrienes and lipoxins formed by initial 5-lipoxygenation of arachidonic acid in animal tissue (12), jasmonic acid and a host of other biologically active compounds formed by initial 9- and 13-lipoxygenation of linoleic and α-linolenic acids in plants (13), and a variety of products formed in algae by initial 12-lipoxygenation of arachidonic and eicosapentaenoic acids (2). Prostaglandins and thromboxanes constitute a group of oxylipins which is formed by initial dioxygenation catalyzed by prostaglandin endoperoxide synthase (14). Oxylipins also may be biosynthesized by cytochrome P-450-catalyzed monooxygenation, in animal (15) as well as in plant tissue (16,17). An additional, recently discovered mechanism of biosynthesis of oxylipins consists of hydroperoxide-dependent epoxidation of fatty acids and hydroxy acids in higher plants (18,19). The present study is concerned with transformation of linoleic acid into hydroxy and ketooctadecadienoic acids in the red alga *Lithothamnion corallioides* by a mechanism which appears to be distinct from previously recognized mechanisms of oxylipin biosynthesis.

The major compound formed from linoleic acid upon incubation with the enzyme preparation of *Lithothamnion* was found to have the structure 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid (Fig. 6). This novel hydroxy acid was related to 13-hydroxyarachidonate and 13-hydroxyeicosapentaenoate recently isolated as natural products from *Lithothamnion* (4), and also to the methyl ester of 11-hydroperoxylinoleic acid, which had been obtained earlier by chemical synthesis (20). The transformation of linoleic acid into 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid demonstrated in the present paper constitutes the first example of an enzymatic reaction in which oxygen is introduced into a *bis*-allylic methylene group of a polyunsaturated fatty acid. Interestingly, the oxygen incorporated originated in water rather than dioxygen. This was an unexpected result, and a number of control experiments were performed in order to verify it. For example, that the lack of incorporation of ^{18}O in 11-hydroxyoctadecadienoic acid biosynthesized from linoleic acid under $^{18}O_2$ was not due to technical problems with the $^{18}O_2$ incubations or with the ^{18}O-gas itself was shown by a control experiment in which linoleic acid was incubated with soybean lipoxygenase using the same atmosphere of $^{18}O_2$

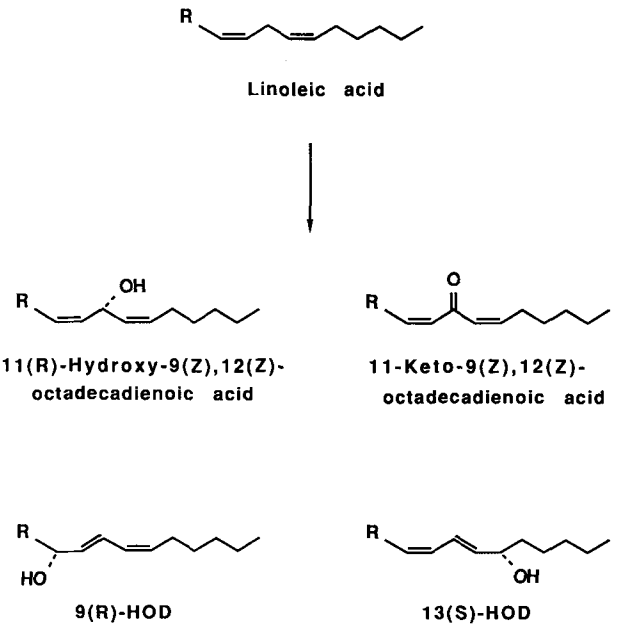

FIG. 6. Transformations of linoleic acid in *Lithothamnion corallioides*. The major enantiomers of the 9- and 13-HOD isolated are shown. R = $(CH_2)_7$-COOH.

as that used for the *Lithothamnion* incubation. The linoleic acid 13-hydroperoxide thus produced showed the expected incorporation of ^{18}O. Furthermore, that the incorporation of ^{18}O into 11-hydroxyoctadecadienoic acid formed from linoleic acid in the presence of $H_2{}^{18}O$ was not due to secondary chemical or enzymatical exchange of ^{18}O into 11-hydroxyoctadecadienoic acid was apparent from an experiment in which 11-hydroxyoctadecadienoic acid was separately incubated with the enzyme preparation in the presence of $H_2{}^{18}O$. No incorporation of ^{18}O in the re-isolated 11-hydroxyoctadecadienoic acid could be detected (Table 1).

The *bis*-allylic alcohol group present in 11-hydroxyoctadecadienoic acid made the compound sensitive to acid. In acidified protic solvents, 11-hydroxyoctadecadienoic acid underwent rapid solvolysis to produce 9- and 13-substituted derivatives having one pair of conjugated double bonds. In aprotic solvents elimination of water took place with the formation of derivatives having a conjugated triene structure. Fatty acids containing conjugated triene and tetraene structures, *e.g.*, elaeostearic and parinaric acids, occur naturally in plants (21). Possibly the facile dehydration of 11-hydroxyoctadecadienoate into conjugated triene acids may have relevance for the biosynthesis of such compounds.

Two other hydroyoctadecadienoates, *i.e.*, 9- and 13-HOD, were obtained as minor compounds following incubation of linoleic acid with *Lithothamnion*. Undoubtedly, part of these compounds was formed non-enzymatically from 11-hydroxyoctadecadienoic acid during the incubation period and the isolation procedure. However, steric analysis showed that the 9- and 13-HOD isolated were not racemates, but due to *ca.* 75% of the *(R)* and *(S)* enantiomers, respectively. It thus seemed likely that *ca.* 50% of the 9- and 13-HOD were formed as the racemic compounds by chemical solvolysis of 11-hydroxyoctadecadienoic acid, while the remaining 50% were formed as the pure *(R)* and *(S)* compounds, respectively, by enzymatic oxidation of linoleic acid. Interestingly, this oxidation occurred by a non-lipoxygenase mechanism as judged by the extensive incorporation of ^{18}O from $H_2{}^{18}O$, but not from $^{18}O_2$, in the hydroxyl groups of 9- and 13-HOD (Table 2). A fourth compound isolated following incubation of linoleic acid, 11-keto-9*(Z)*,12*(Z)*-octadecadienoic acid, was apparently formed by enzymatic oxidation of 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid (Fig. 5B). As expected from its formation from 11-hydroxyoctadecadienoic acid, the oxygen of the C-11 keto group of 11-ketooctadecadienoic acid was derived from water, and not from dioxygen (Table 2).

The transformation of linoleic acid into 11*(R)*-hydroxy-9*(Z)*,12*(Z)*-octadecadienoic acid was oxygen-dependent (Table 3), involved incorporation of 1 atom of oxygen from water (Table 2), and was not inhibitable, or only moderately so, by lipoxygenase and cytochrome P-450 inhibitors (Table 3). If the transformation occurred by a single enzymatic step it followed, per definition, that the enzyme involved was not an oxygenase. It may be speculated that an oxidase was responsible for the conversion; however, it is apparent that further studies on the enzyme(s) involved in the transformation are necessary before any conclusions as to the mechanism may be drawn. Such work is in progress in our laboratory.

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Metabolism of [1-^{14}C]Docosahexaenoate (22:6n-3), [1-^{14}C]Eicosapentaenoate (20:5n-3) and [1-^{14}C]Linolenate (18:3n-3) in Brain Cells from Juvenile Turbot *Scophthalmus maximus*

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The incorporation of [1-^{14}C]18:3n-3 (LNA) and [1-^{14}C]-22:6n-3 (DHA), and the metabolism *via* the desaturase/elongase pathways of [1-^{14}C]LNA and [1-^{14}C]20:5n-3 (EPA) were studied in brain cells from newly-weaned (1-month-old) and 4-month-old turbot. The rank order of the extent of net incorporation of both LNA and DHA into glycerophospholipids was total diradyl glycerophosphocholines (CPL) > total diradyl glycerophosphoethanolamines (EPL) > phosphatidylserine (PS) and phosphatidylinositol (PI) and was independent of the polyunsaturated fatty acid added, the age of the fish and the time of incubation. However, the rate of incorporation of LNA into total lipid, CPL, EPL and PS was significantly greater than the rate of incorporation of DHA, and there was a significantly greater amount of DHA incorporated into EPL than LNA. There was no significant difference between the amounts of LNA and DHA incorporated into total lipid, CPL, PS and PI. Therefore, little preferential uptake and incorporation of DHA into brain cells was apparent. In 24-h incubations, on average 1.1% and 8.5% of radioactivity from [1-^{14}C]LNA and [1-^{14}C]EPA, respectively, were recovered in the DHA fraction. Therefore, LNA cannot contribute significantly to brain DHA levels in the turbot but EPA can. There were no significant differences between the amounts of radioactivity from either [1-^{14}C]LNA or [1-^{14}C]EPA recovered in the individual products/intermediates of the desaturase pathways in brain cells from 30-day-old and 120-day-old turbot.
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Neural tissues from all vertebrates are characterized by high levels of n-3 polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA; 22:6n-3) (1). Despite the high level of n-3 PUFA in all their tissues, this phenomenon is evident also in fish brain and retina where total n-3 PUFA and DHA levels are elevated above the levels found in non-neural tissues (2). DHA is tenaciously retained by neural tissues during dietary deprivation of n-3 PUFA in mammals (3) and two generations of deprivation are required to reduce significantly DHA levels in brain and retina (4-7). Depletion of neural DHA levels was accompanied by alterations in physiological parameters and functional defects such as reduced visual acuity and impaired learning abilities (4-7). This research established a critical role for DHA in neural tissue functions in mammals (8,9) and the nutritional importance of n-3 PUFA during neural development in mammals is now increasingly recognized (10,11).

It is unclear how much brain DHA is derived from endogenous biosynthesis from α-linolenic acid (LNA; 18:3n-3) in the brain and how much is obtained preformed from the liver or dietary sources (8). *In vivo* production of DHA from LNA has been demonstrated in rat brain (12) but other evidence suggests that much of the DHA in mammalian brains results from the accumulation of preformed DHA during neural development (13-15). Some of this DHA may be obtained directly as maternal DHA and some may be produced in the liver from maternal LNA (16). Until recently this has been a virtually unstudied area in fish. However, we have recently demonstrated that DHA accumulates rapidly in brains from farmed turbot in the period immediately following weaning onto dry pellet food relatively rich in DHA (17). Prior to weaning from a diet relatively deficient in DHA, the turbot brains have low levels of DHA, so that the increase in brain DHA levels appears to be dependent upon the provision of dietary DHA (18).

Previously, it was shown that turbot require dietary C_{20} and C_{22} PUFA for optimal growth (19) and that turbot, aged 10 mon, could not convert dietary [1-^{14}C]LNA to DHA (20). Deficient Δ5 fatty acid desaturase activity and/or C_{18}-C_{20} elongase has been demonstrated in turbot in both intact fish (21) and cells in culture (22,23), although the Δ4 fatty acid desaturase responsible for the conversion of eicosapentaenoic acid (EPA; 20:5n-3) to DHA appeared to be active in the systems studied. Brain tissue or cells have not been investigated in any of the previous studies.

In the present study, we have investigated n-3 PUFA metabolism in brain cells from rapidly growing and developing turbot. Specifically, we have studied the metabolism of [1-^{14}C]DHA, [1-^{14}C]EPA and [1-^{14}C]LNA in brain cells from newly-weaned (1-month-old) and 4-month-old turbot. We aimed to determine i) if there was a differential uptake and incorporation of DHA into brain cells, ii) the extent to which LNA and EPA could contribute to brain DHA levels and iii) if these processes were affected by age and development of the fish.

MATERIALS AND METHODS

Experimental animals. Juvenile turbot (*S. maximus*) were obtained from a commercial turbot hatchery (Golden Sea Produce, Hunterston, Ayrshire, Scotland) and were maintained in 2-m circular tanks supplied with filtered, recirculating sea water. Temperature was 10 ± 2°C, and the fish were given commercial diets as used in the hatchery

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Abbreviations: ANOVA, analysis of variance; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CPL, total diradyl glycerophosphocholines; DHA, 4,7,10,13,16,19-docosahexaenoic acid (22:6n-3); EPA, 5,8,11,14,17-eicosapentaenoic acid (20:5n-3); EPL, total diradyl glycerophosphoethanolamines; GC, gas chromatography; HBSS, Hank's balanced salt solution; HPTLC, high-performance thin-layer chromatography; LNA, 9,12,15-octadecatrienoic acid, α-linolenic acid (18:3n-3); PI, (diacyl)phosphatidylinositols; PS, (diacyl)phosphatidylserines; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

(17,18). Two ages of turbot were studied. The younger group were newly-weaned fish of approximate age 30 days and the older group were fish that had been weaned for 3 mon (approximately 120 days old).

Preparation of isolated brain cell suspensions. Fish were killed by cutting the spinal cord immediately posterior to the brain. Entire brains were removed into ice-cold Hank's balanced salt solution (Ca^{2+}- and Mg^{2+}-free and supplemented with 1.75% NaCl)(HBSS) and finely chopped. A mixed brain cell suspension was produced by sequential mechanical sieving of the chopped tissue through sterile nylon gauzes of 100 and 30 μm essential as described by McCarthy and De Vellis (24). The suspension was centrifuged at 300 × *g* for 10 min at 4°C and the cell pellet resuspended in Dulbecco's modification of Eagle's medium supplemented with 4 mM glutamine, antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin) and 0.35% NaCl. Cells were resuspended in 6.5 mL and 3 mL of medium for incorporation time-course and metabolic experiments, respectively. The numbers of fish used in each incorporation time-course and metabolic experiment were 36 and 18, respectively, for 30-day turbot. For 120-day-old turbot the numbers of fish per experiment as above were 15 and 7, respectively. Viability was assessed by trypan blue exclusion at the beginning and end of each experiment (6 h for incorporation experiments and 24 h for metabolic experiments). The cell numbers remained constant throughout the incubation periods but viability of the cells decreased from >86% upon preparation of the suspension to <72% after 6 h incubation (Table 1). The viability was not affected by a further 18 h incubation.

Incubation of brain cells with ^{14}C-labelled PUFA. For incorporation experiments, 2.75 mL of brain cell suspension had 0.6 μCi of either [1-^{14}C]LNA or [1-^{14}C]DHA added, carrier-free, in 15 μL ethanol (final conc., 4.0–4.5 μM PUFA and 0.55% ethanol). For metabolic experiments, 1 mL of suspension had 0.25 μCi of either [1-^{14}C]LNA or [1-^{14}C]EPA added, carrier free in 6 μL ethanol (final conc. 4–5 μM PUFA and 0.6% ethanol). Incubations were performed in a shaking water bath at 15°C.

Lipid extraction. Samples of suspension (0.5 mL and 1 mL in incorporation and metabolic experiments, respectively) had 5 mL ice-cold HBSS containing 1% fatty acid-free bovine serum albumin (BSA) added. After mixing, the samples were centrifuged at 500 × *g* for 2 min and the supernatants aspirated. The cells were washed once more with HBSS as above and total lipid extracted essentially according to Folch *et al.* (25) as described previously (26). Lipid content of each suspension was determined in a 0.5 mL sample extracted in parallel, with the total lipid content determined gravimetrically.

Incorporation of radioactivity into glycerophospholipid classes. Samples of total lipid had 50 μg of unlabelled turbot brain lipid added as carrier and were then applied in 1-cm streaks to high-performance thin-layer chromatography (HPTLC) plates. Polar lipid classes were separated using methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol) (27). Lipids were visualized by brief exposure to iodine vapor, marked, and the iodine removed under vacuum. Individual classes were scraped into scintillation minivials, 2.5 mL of Ecoscint A (National Diagnostics, Manville, NJ) added, and radioactivity determined using a Packard 2000CA Tri-Carb liquid scintillation analyzer (Downers Grove, IL).

TABLE 1

Protein and Lipid Contents, and Viability of Brain Cells During 24-h Incubations of Brain Cell Suspensions from 30- and 120-day-old Turbot *Scophthalmus maximus*[a]

	Age of turbot	
	30-days-old	120-days-old
Protein content (mg/mL)	0.91 ± 0.14	0.81 ± 0.13
Lipid content (mg/mL)	0.65 ± 0.08	1.33 ± 0.42
Lipid /protein ratio	0.71 ± 0.04	1.64 ± 0.22
	Time of incubation	
	0 h	6 h	24 h
Cell number (10^6/mL)	1.4 ± 0.1	1.6 ± 0.2	1.6 ± 0.1
Cell viability (%)	86.2 ± 2.6	71.7 ± 3.4	71.3 ± 3.5

[a]Values are means ± SD of at least six suspensions.

Incorporation of radioactivity into PUFA. Fatty acid methyl esters from total lipid were prepared by acid-catalyzed transmethylation at 50°C for 16 h (28) and extracted and purified as described previously (2). Methyl esters were separated by a combination of argentation thin-layer chromatography (TLC) and radio gas chromatography (GC), and radioactivity in the fatty acid methyl ester fractions was determined as described in detail previously (29,30). All solvents contained 0.01% butylated hydroxytoluene (BHT) as antioxidant.

Protein content. Samples of 0.5 mL of each suspension were taken into NaOH/sodium dodecylsulfate for protein determination essentially according to Lowry *et al.* (31).

Statistical analysis. All results are the means of at least three experiments ± 1 SD. The data were checked for homogeneity of the variances by the Bartlett test and, where necessary, the data were log-transformed before further statistical analysis. The kinetics of the incorporation of [1-^{14}C]LNA and [1-^{14}C]DHA into various lipids were determined by non-linear regression analysis and fitted to the 1st order rate equation $y = A(1\text{-}e^{-kt})$, where A is the limit and k is the rate constant. Differences between means were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The effects on the kinetics of incorporation due to different [^{14}C]PUFA in brain cells from turbot of different ages were analyzed by a model I two-way ANOVA.

Materials. [1-^{14}C]PUFA (all 50–55 mCi/mmol and 99% pure were obtained from NEN, DuPont (U.K.) Ltd. (Stevenage, U.K.). BHT, fatty acid-free BSA and silver nitrate were from Sigma Chemical Co. Ltd. (Poole, U.K.). Medium, HBSS, glutamine and antibiotics were obtained from Northumbria Biologicals Ltd. (Northumberland, U.K.). TLC (20 × 20 cm × 0.25 mm) and HPTLC (10 × 10 cm × 0.15 mm) plates, precoated with silica gel 60 (without fluorescent indicator), were obtained from Merck (Darmstadt, Germany). All solvents were HPLC grade and were obtained from Rathburn Chemicals (Walkerburn, Scotland).

RESULTS

The lipid/protein ratio was significantly greater in the older fish indicating that there was considerable bio-

bsynthesis and/or accumulation of lipid in the turbot brains during this period of development (Table 1).

The time-courses for the incorporation of [1-^{14}C]LNA and [1-^{14}C]DHA into brain cells from 30- and 120-day-old turbot are shown in Figure 1. Under the conditions of the experiments, the incorporations had reached maxima by 2 h in the case of LNA and 4 h for DHA. The time courses for the incorporation of [1-^{14}C]LNA and [1-^{14}C]DHA into individual glycerophospholipids are shown in Figures 2 and 3, respectively. The rank order of incorporation into the glycerophospholipids was total diradyl glycerophosphocholines (CPL) > total diradyl glycerophosphoethanolamines (EPL) > (diacyl)phosphatidylserine (PS) and (diacyl)phosphatidylinositol (PI), and was independent of ^{14}C-labelled PUFA, age of the fish, and time of incubation (Figs. 2 and 3).

The kinetics of the incorporation of [1-^{14}C]LNA and [1-^{14}C]DHA into total lipid and individual glycerophospholipids were determined and analyzed statistically. The rate of incorporation of [1-^{14}C]LNA into total lipid, CPL, EPL and PS was significantly greater than the rate of incorporation of [1-^{14}C]DHA (Table 2, Figs. 1-3). This was also apparent for PI, but as some of these data were only in duplicate the statistical analysis could not be performed. In almost every case the rates of incorporation were lower in brain cells from the older fish, but the errors involved made these data not statistically significant.

The ANOVA analysis showed that, under the conditions of the experiments, there was no significant difference between the amounts of [1-^{14}C]LNA and [1-^{14}C]DHA incorporated into total lipid, CPL, PS and PI (Table 2). However, there was significantly greater incorporation of [1-^{14}C]DHA into EPL than [1-^{14}C]LNA. In particular, the incorporation of [1-^{14}C]DHA into EPL in brain cells from 30-day turbot was more than double the incorporation of [1-^{14}C]LNA into EPL in both 30- and 120-day-old turbot (Table 2, Figs. 1-3). As with the rate of incorporation, although the amount of incorporation of both labelled PUFA generally tended to be lower in brain cells from older fish, there was no statistical significance to the differences. The two-way ANOVA showed that there was no interrelationship between the effects of PUFA and age of the fish (Table 2).

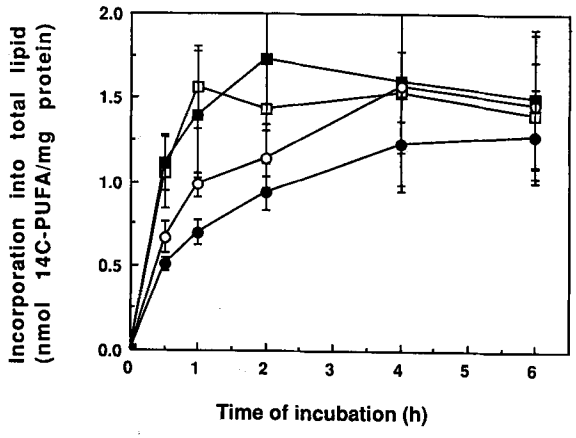

FIG. 1. Incorporation of [^{14}C]18:3n-3 and [^{14}C]22:6n-3 into total lipid of brain cells from turbot at 30 days and 120 days after hatching. Isotopes were added to cell suspensions to final fatty acid concentrations of 4.0–4.5 μM and incubations were performed at 15°C. Samples were withdrawn and analyzed as described in Materials and Methods. Results are means ± SD (n = 3). (□,■)[^{14}C]18:3n-3; (○,●)[^{14}C]22:6n-3. Open symbols, 30 days; closed symbols, 120 days.

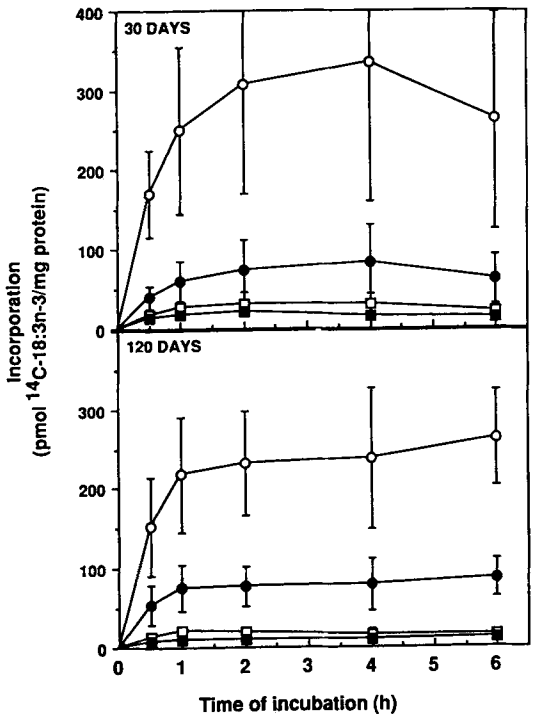

FIG. 2. Incorporation of [^{14}C]18:3n-3 into glycerophospholipid classes in brain cells from turbot at 30 days and 120 days after hatching. Incubation conditions as in Figure 1. Analyses performed as described in Materials and Methods. Results are means ± SD (n = 3). (○), total diradyl glycerophosphocholine; (●), total diradyl glycerophosphoethanolamine; (□), phosphatidylserine; (■), phosphatidylinositol.

The metabolism of [1-^{14}C]LNA and [1-^{14}C]EPA *via* the desaturase pathways over a 24-h period are shown in Table 3. In brain cells from 30-day-old turbot, only 5% of radioactivity from [1-^{14}C]LNA was recovered in products/intermediates of the normal desaturase/elongase pathway with only half of that percentage appearing as products beyond the Δ5 desaturase step (Table 3). The dead-end product 20:3n-3 accounted for the largest single percentage of metabolized [1-^{14}C]LNA. Over 12% of incorporated [1-^{14}C]EPA was chain elongated to 22:5n-3 with 7% being further desaturated *via* Δ4 desaturase activity to DHA in brain cells from 30-day-old turbot (Table 3).

There were lower percentages (significantly so with labelled LNA) of radioactivity recovered in the precursor fatty acid fractions in the brain cells from 120-day-old turbot (Table 3). However, there were no significant differences between the amounts of radioactivity from either labelled PUFA recovered in the individual products/intermediates of the desaturase pathways in brain cells from 30-day-old and 120-day-old turbot.

DISCUSSION

The results of the present study strongly suggest that there is no preferential uptake and incorporation of DHA into juvenile turbot brain cells. Furthermore, under

TABLE 2

Incorporation of [^{14}C]18:3n-3 and [^{14}C]22:6n-3 into Lipids in Brain Cells from Turbot *Scophthalmus maximus*[a]

		[^{14}C]Polyunsaturated fatty acid added				Significance level		
		[^{14}C]18:3n-3		[^{14}C]22:6n-3				
		Age of turbot 30 days	Age of turbot 120 days	Age of turbot 30 days	Age of turbot 120 days	FA	AGE	FA × AGE
TL	A	1500.6 ± 380.3	1605.6 ± 415.3	1511.9 ± 340.9	1279.2 ± 301.5	n.s.	n.s.	n.s.
	k	3.5 ± 1.6^{a,b}	2.5 ± 0.4^{a}	1.1 ± 0.4^{b}	0.9 ± 0.3^{b}	0.002	n.s.	n.s.
CPL	A	304.5 ± 154.1	248.0 ± 61.9	259.1 ± 57.8	181.9 ± 41.2	n.s.	n.s.	n.s.
	k	2.2 ± 0.8^{a,b}	1.9 ± 0.5^{a}	0.7 ± 0.1^{b,c}	0.5 ± 0.1^{c}	0.0002	n.s.	n.s.
EPL	A	73.8 ± 39.5^{a}	82.2 ± 24.1^{a}	175.1 ± 32.3^{b}	105.5 ± 33.9^{a,b}	0.028	n.s.	n.s.
	k	2.5 ± 1.4^{a,b}	2.2 ± 0.7^{a}	0.5 ± 0.0^{b}	0.6 ± 0.2^{b}	0.001	n.s.	n.s.
PS	A	28.8 ± 11.5	17.9 ± 4.6	31.6 ± 16.2	12.7 ± 5.3	n.s.	n.s.	n.s.
	k	2.2 ± 0.5^{a,b}	3.4 ± 0.7^{a}	1.2 ± 0.3^{b,c}	0.8 ± 0.3^{c}	0.001	n.s.	n.s.
PI	A	17.2 ± 4.1	11.5 ± 0.9	31.4 ± 3.5	16.7 ± 2.3	n.d.	n.d.	n.d.
	k	3.9 ± 0.8	2.5 ± 0.9	1.3 ± 0.7	0.8 ± 0.2	n.d.	n.d.	n.d.

[a]Data fitted to 1st order rate equations defined by A (limit, pmol [^{14}C]PUFA/mg protein) and k (rate constant, 1/h). For statistical treatments see Materials and Methods section. Values are means ± SD of three samples with the exception of some values in PI where means of duplicates ± range are shown. Values within a given row not bearing the same superscript letter are significantly different at $P < 0.05$. If no superscript appears, values are not different. AGE, age effect; FA, fatty acid effect; CPL, total diradyl glycerophosphocholines; EPL, total diradyl glycerophosphoethanolamines; n.d., not determined; n.s., not significant; PI, phosphatidylinositol; PS, phosphatidylserine; TL, total lipid.

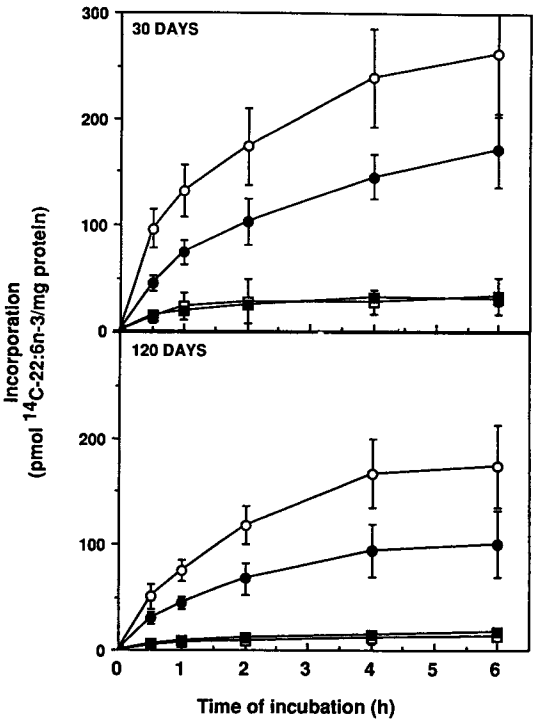

FIG. 3. Incorporation of [^{14}C]22:6n-3 into glycerophospholipid classes in brain cells from turbot at 30 days and 120 days after hatching. Incubation conditions as in Figure 1. Analyses performed as described in Materials and Methods. Results are means ± SD (n = 3). (○), total diradyl glycerophosphocholine; (●), total diradyl glycerophosphoethanolamine; (□), phosphatidylserine; (■), phosphatidylinositol.

identical experimental conditions, although the initial rate of incorporation of LNA was greater than that of DHA, both PUFA were incorporated to approximately the same final level. No data on the incorporation of DHA into fish brains or brain cells are available for comparison. Furthermore, although the rapid and specific accumulation of DHA (and 20:4n-6) into mammalian brains during development is well established (13–15,32), there are few data comparing directly the incorporation of different PUFA into brains or brain cells in mammals. However,

TABLE 3

Metabolism *via* the Desaturase Pathway of [^{14}C]18:3n-3 and [^{14}C]20:5n-3 in Brain Cells from Turbot *Scophthalmus maximus*[a]

	[^{14}C]Polyunsaturated fatty acid added			
	[^{14}C]18:3n-3		[^{14}C]20:5n-3	
	Age of turbot 30 days	Age of turbot 120 days	Age of turbot 30 days	Age of turbot 120 days
Fraction				
18:3	91.9 ± 2.5	86.7 ± 1.4^{b}	n.d.	n.d.
20:3	3.8 ± 2.5	5.8 ± 0.6	n.d.	n.d.
18:4	1.1 ± 0.9	2.2 ± 0.7	n.d.	n.d.
20:4	1.3 ± 0.7	2.1 ± 0.7	n.d.	n.d.
20:5	1.2 ± 0.4	1.4 ± 0.8	87.6 ± 2.4	79.4 ± 3.5
22:5	0.6 ± 0.2	0.6 ± 0.3	5.3 ± 1.1	10.6 ± 6.1
22:6	0.9 ± 0.4	1.3 ± 0.4	7.1 ± 2.0	10.0 ± 7.8

[a]Values show the radioactivity found in each fatty acid fraction expressed as a percentage of total radioactivity recovered and are means ± SD (n = 3).

[b]The differences between values at 30 and 120 days were significantly different ($P < 0.05$) as determined by Student's *t*-test; n.d., not determined.

the uptake of i.v.-injected ^{14}C-labelled DHA into brains of 15-day-old rats was greater than the uptake of LNA, 18:2n-6 and 16:0 (33). Similarly, in primary cultures of rat neurons, the incorporation of DHA exceeded that of LNA (34).

The absolute level of incorporation of both LNA and DHA was lower in the turbot brain cells than previously found in mammalian systems although there is considerable variation between different studies. Yavin and Menkes (35) reported an incorporation of approximately 4–5 nmol [1-^{14}C]LNA/mg protein after 3 h incubation in cultures of dissociated rat cerebrum cells, about 3 times the level of incorporation found with [1-^{14}C]LNA in the turbot brain cell suspensions in the present study. In a recent study, [1-^{14}C]DHA and [1-^{14}C]LNA were incorporated into rat neurons in primary culture to levels of 150 and 75 mmol/mg protein, respectively, after 8 h incubation (34). These differences could be the results of different concentrations of labelled PUFA (7 μM and 18 μM, respectively, in the previous studies compared with 4.0–4.5 μM in the present study), incubation temperature (37°C with the mammalian cells and 15°C with the turbot cells), cell profiles in the different preparations, or inherent differences between mammalian and fish cells.

The distribution of incorporated [1-^{14}C]DHA and [1-^{14}C]LNA in glycerophospholipids in the present study was similar to that obtained previously in other fish cells and several mammalian neural cells. The rank order of the distribution of incorporated [1-^{14}C]LNA was CPL > EPL > PS/PI in primary cultures of rainbow trout astrocytes (29) and established cell cultures from rainbow trout gonad, Atlantic salmon fry and turbot fin (30). The incorporation of DHA was not investigated in these studies. In turbot injected i.v. with ^{14}C-labelled DHA and LNA, the pattern of incorporation for both PUFA was CPL > EPL > PS/PI (21). This rank order was also found with the incorporation of ^{14}C-labelled LNA in primary rat astroblast cultures (36) and in cultured dissociated cells from rat cerebrum (35). In rats injected i.v. with ^{14}C-labelled DHA, 80–85% of radioactivity in the brain after 4 h was recovered in CPL/EPL (37).

Although the distribution of [1-^{14}C]DHA and [1-^{14}C]-LNA between the glycerophospholipids was qualitatively similar in the present study, quantitatively there was a difference in that significantly more DHA was incorporated into EPL. Previously, it was shown in rats with intracerebral injections of ^{14}C-labelled DHA and ^{3}H-labelled 20:4n-6 that DHA was selectively incorporated into EPL compared to 20:4n-6 (38). The relatively high concentration of EPL in neural tissues compared with non-neural tissues in both mammals and fish (2) combined with the high concentration of DHA in EPL means that the bulk of neural DHA is present in EPL. This may imply some special role for EPL in neural tissue functions.

The present study showed that LNA could not contribute significantly to DHA levels in juvenile turbot brains. This was, perhaps, expected as previous work on turbot showed that little or no labelled DHA was produced from injected ^{14}C-labelled LNA in juveniles (21) or 10-month-old fish (20). Similarly, only 1% on average of ^{14}C-labelled LNA was converted to DHA in turbot fin cells in culture (30). However, significant amounts (6–7%) of labelled DHA were produced from ^{14}C-labelled EPA in the present study on turbot brain cells and approximately 14% of radioactivity from injected ^{14}C-labelled EPA was recovered in the DHA fraction in juvenile turbot after 48 h (21). Overall, the data from the present study using [1-^{14}C]EPA and [1-^{14}C]LNA show that juvenile turbot brain expresses the same range of fatty acid desaturase activities as found previously in whole fish and in fin cell cultures, *i.e.* Δ6 and Δ4 desaturase activities are expressed whereas Δ5 desaturase activity is very low or lacking.

There are no data from neural tissues of fish that do not lack Δ5 desaturase activity, *i.e.* trout or salmon, with which to compare the turbot data from the present study. However, in rats, radioactivity from intracranially injected ^{14}C-labelled LNA was recovered in DHA of the brain (12). In cultures of dissociated cells from rat cerebrum incubated with ^{14}C-labelled LNA, 10–25% of the radioactivity was recovered in DHA (35,39). In rat brain, the conversion of LNA to DHA has been localized in the astroglial cell fraction (34), whereas rat neurons and mouse brain microvascular endothelial cells both lacked Δ4 desaturase activity (34,40).

Desaturase activities are known to vary, generally decreasing, with increasing age of animals (41–44). In prenatal rat pups injected intracranially with ^{14}C-labelled LNA, only 1% of the radioactivity in the brain was recovered in DHA (45). Similarly, in brain microsomes from neonatal monkeys (and up to 2 years old), very little DHA was produced from EPA (46). In the present study, significant amounts of labelled DHA were produced in the turbot brain cells, but there was no difference between 1-month-old and 4-month-old turbot in the metabolism of either [1-^{14}C]EPA or [1-^{14}C]LNA *via* the desaturase pathways. In contrast, in pre-weaned turbot of <1 mon of age, there was no evidence for significant conversion of EPA to DHA *in vivo* (17,18) perhaps reflecting a lower level of Δ4 desaturase activity in pre-juvenile and, possibly, larval turbot.

The present study has strongly suggested that the accumulation of DHA in turbot brain during development is not due to specific uptake and incorporation of DHA and that endogenous desaturation of LNA cannot contribute to brain DHA levels. This confirms our previous conclusions, from studies on lipid and fatty acid compositions of developing turbot brain, that the level of DHA in the brain was markedly dependent upon the level of DHA in the diet (17,18). We conclude, therefore, that a strictly carnivorous marine fish such as the turbot is heavily dependent on a substantial dietary input of DHA for normal neural and visual development. The implications of this conclusion for precise prey selection by early larval turbot in nature, and for the provision of an adequate diet for larval turbot in mariculture, are considerable.

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5c,11c,14c-Eicosatrienoic Acid and 5c,11c,14c,17c-Eicosatetraenoic Acid of *Biota orientalis* Seed Oil Affect Lipid Metabolism in the Rat

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The effects of 5c,11c,14c-eicosatrienoic acid (20:3BSO) and 5c,11c,14c,17c-eicosatetraenoic acid (20:4BSO), polyunsaturated fatty acids (PUFA) contained in *Biota orientalis* seed oil (BSO), on lipid metabolism in rats were compared to the effects of fats rich in linoleic acid (LA) or α-linolenic acid (ALA) under similar conditions. The potential effect of ethyl 20:4BSO as an essential fatty acid also was examined in comparison with the ethyl esters of LA, ALA and γ-linolenic acid (GLA). BSO- and ALA-rich fat decreased the concentration of plasma total cholesterol, high density lipoprotein cholesterol, triglyceride and phospholipid as compared to LA-rich fat. BSO was more effective in reducing plasma cholesterol concentrations than was the ALA-rich fat. Dietary BSO markedly decreased the hepatic triglyceride concentration as compared to the LA-rich or ALA-rich fats. Aortic production of prostaglandin I_2 tended to decrease in rats fed BSO or ALA-rich fat compared to those fed the LA-rich fat. Adenosine diphosphate-induced platelet aggregation was similar in the three groups. The proportion of arachidonic acid (AA) in liver phosphatidylcholine (PC) of rats fed BSO was lowest compared to that of rats fed ALA-rich or LA-rich fats. Administration of 20:4BSO, ALA or GLA to essential fatty acid-deficient rats decreased the ratio of 20:3n-9 to AA in liver PC to the same extent; administration of LA was more effective. The results indicate that the effects of specific PUFA contained in BSO on lipid metabolism are different from those of LA and ALA. It is also suggested that 20:4BSO may exhibit some essential fatty acid effects.

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Biota orientalis seeds have been used for many years in Chinese medicine. The oil extracted from the seeds contains anomalous n-6 and n-3 polyunsaturated fatty acids (PUFA), 5c,11c,14c-eicosatrienoic (20:3BSO) and 5c,11c,14c,17c-eicosatetraenoic (20:4BSO) acids (1,2). While 20:3BSO lacks the double bond at position 8 of arachidonic acid (AA), 20:4BSO lacks the double bond at position 8 of eicosapentaenoic acid (EPA). Although different PUFA are known to have different metabolic effects in mammals (3), no information is available on the biological effects of the PUFA occurring in BSO. In the present study, we examined the effect of specific PUFA occurring in *Biota orientalis* seed oil (BSO) on serum and liver lipid concentrations, platelet aggregation and prostaglandin formation in rats. The effect of 20:4BSO as an essential fatty acid also was assessed in essential fatty acid (EFA)-deficient rats.

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Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; ALA, α-linolenic acid; BSO, *Biota orientalis* seed oil; 20:3BSO, 5c,11c,14c-eicosatrienoic acid (n-3); EFA, essential fatty acid(s); EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; GLC, gas-liquid chromatography; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LA, linoleic acid; PC, phosphatidylcholine(s); PE, phosphatidylethanolamine(s); PGI_2, prostacyclin; PUFA, polyunsaturated fatty acid(s); TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Materials. BSO was extracted with hexane from *Biota orientalis* seed purchased from herb shops in Guangzhou, China, and was purified to a grade of refined edible oils by the general purification processes through the courtesy of Nihon Yushi Co. (Tokyo, Japan). Linseed, high oleic safflower and safflower oils were obtained from Nisshin Oil Mills Co. (Tokyo, Japan), Fuji Oil Co. (Tokyo, Japan) and Linol-Yushi (Tokyo, Japan), respectively. Purified ethyl linoleic acid (LA), ethyl α-linolenic acid (ALA) and ethyl γ-linolenic acid (GLA) (>98% purity) were obtained from Idemitsu Chemicals (Tokyo, Japan). After BSO was saponified and the fatty acids were converted to ethyl esters, ethyl 20:4BSO was separated and purified by preparative high-performance liquid chromatography (HPLC) at Nisshin Oil Mills Co. The preparation was 85.3% pure and contained 12.0% ALA and 2.7% LA as impurities.

Animals and diets. Male Sprague-Dawley rats (4-wk-old in Exp. 1, and 3-wk-old in Exp. 2) obtained from Seiwa Experimental Animals (Fukuoka, Japan) were housed individually and kept in a room at controlled temperature (22-24°C) and lighting (8:00 a.m. to 8:00 p.m.). In Exp. 1, the rats were divided into 3 groups of 6 animals each and fed the experimental diets for 24 days. The basal diet was prepared according to the formula recommended by the American Institute of Nutrition (4) and contained (by weight percent): Casein 20, fat 10, vitamin mixture 1.0, mineral mixture 3.5, choline bitartrate 0.2, DL-methionine 0.3, cholesterol 0.5, sodium cholate 0.125, cellulose 5.0, corn starch 15 and sucrose to 100. A mixture of 90% safflower oil and 10% high oleic safflower oil was used as the LA-rich fat, and a mixture of 85% linseed oil and 15% safflower oil was used as the ALA-rich fat. The fatty acid compositions of the fats are shown in Table 1.

TABLE 1

Fatty Acid Composition of Dietary Fat[a]

Dietary fat	Fatty acids (weight %)						
	16:0	18:0	18:1	18:2n-6	18:3n-3	20:3BSO	20:4BSO
ALA[b]	4.9	2.8	17.3	25.7	49.1	—	—
LA[c]	6.1	2.4	18.4	72.6	—	—	—
BSO	5.2	4.6	17.5	23.9	32.1	4.8	8.9

[a]20:3BSO, 5c,11c,14c-eicosatrienoic acid; and 20:4BSO, 5c,11c,14c,17c-eicosatetraenoic acid.

[b]ALA-rich fat.

[c]LA-rich fat.

In Exp. 2, 70 rats were divided into 2 groups. Twelve rats were fed the basal diet supplemented with 1.4% safflower oil, and 58 rats were given a fat-free diet. The composition of the basal diet was the same as in Exp. 1, except for the exclusion of fat, cholesterol and sodium cholate. After 66 days on the diets, 6 rats given the safflower oil-supplemented diet and 5 rats fed the fat-free diet were killed. The rest of the rats, fed a fat-free diet, were divided into five groups. Four groups of 12 rats each were given 100 mg/day (orally) of either ethyl LA, ethyl ALA, ethyl GLA or ethyl 20:4BSO, and 6 rats in each group were killed after consecutive administration of the ethyl esters for 3 and 10 days while they were continuously given a fat-free diet. Six rats fed the safflower oil-supplemented diet and five rats fed the fat-free diet were given a glucose solution which contained the same amount of calories as 100 mg of fatty acid ethyl esters, and were killed after 10 days. In both experiments, after 7-h fasting from 6:00 a.m. to 1:00 p.m., rats were anesthetized with diethyl ether. Blood (9 mL) was collected from the abdominal aorta in a syringe containing 1 mL of 3.8% trisodium citrate and indomethacin (final concentration of 0.1 mM).

Lipid analyses. Liver, plasma and epididymal adipose tissue were extracted in 20 vol of chloroform-methanol (2:1, vol/vol) according to Folch *et al.* (5). Liver and plasma cholesterol, triglyceride and phospholipid were measured as described previously (6). Liver phosphatidylcholine (PC), phosphatidylethanolamine (PE), triglyceride and cholesteryl ester, as well as plasma cholesteryl ester and triglyceride were separated by thin-layer chromatography (TLC), and the fatty acid compositions were analyzed by gas-liquid chromatography (GLC) on a SILAR 10C column (Nihon Kuromato Kogyo, Tokyo, Japan) (7). Fatty acid compositions of total lipids in epididymal adipose tissue also were analyzed by GLC.

Prostaglandin analyses. The thoracic aortas were incubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) at 25°C for 30 min and the concentration of 6-keto prostaglandin $F_{1\alpha}$ in the medium was measured with a commercially available radioimmunoassay kit (NEK-008, New England Nuclear, Boston, MA) (8). The concentration of 11-dehydro-thromboxane B_2 in plasma also was measured with a commercial kit (NEK-042, New England Nuclear) as described previously (9).

Statistical analysis. All data were analyzed by Duncan's new multiple range test (10).

RESULTS

Experiment 1

Body weight, food intake and liver weight. As shown in Table 2, no significant differences were found in body weight gain, food intake and relative liver weight among the three groups.

Plasma and liver lipids. The concentration of plasma and liver lipids is shown in Table 3. The concentration of plasma total cholesterol was lowest in the BSO group and highest in the LA-rich fat group, and intermediate in the ALA-rich fat group. The difference between each group was significant. The concentrations of plasma high density lipoprotein (HDL) cholesterol and phospholipid were significantly lower in rats fed ALA-rich fat or BSO than in those fed LA-rich fat. A similar tendency was observed in the concentration of plasma triglyceride.

The concentration of liver triglyceride in the BSO group was significantly decreased by more than 50% relative to the ALA-rich or LA-rich fat groups. The concentration of liver cholesterol was similar in the three groups, whereas that of liver phospholipid was higher in the BSO group than in the other two groups.

Prostaglandin I_2 and platelet aggregation. Aortic production of prostacyclin (PGI_2) measured as 6-keto-prostaglandin $F_{1\alpha}$ significantly decreased in rats fed BSO as compared to those fed LA-rich fat (Fig. 1). The production in rats fed ALA-rich oil also tended to be lower than in those fed LA-rich fat. There was no difference in the

TABLE 2

Body Weight, Food Intake and Relative Liver Weight[a]

Groups	Body weight (g)		Food intake (g/day)	Liver weight (g/100 g body weight)
	Initial	Final		
ALA[b]	114 ± 4	169 ± 6	18.1 ± 0.6	7.1 ± 0.3
LA[c]	115 ± 3	171 ± 5	19.1 ± 0.5	7.1 ± 0.4
BSO	115 ± 2	162 ± 5	18.2 ± 0.5	6.8 ± 0.2

[a]Data are means ± SE of six rats.
[b]ALA-rich fat.
[c]LA-rich fat.

TABLE 3

Concentration of Lipids in Plasma and Liver[a]

Group	Plasma (mg/100 mL)				Liver (mg/g)		
	CHOL	HDL-CHOL	TG	PL	CHOL	TG	PL
ALA[b]	112 ± 8[c]	29.4 ± 3.3[c]	19.1 ± 2.5[c]	124 ± 6[c]	64.9 ± 4.7	55.6 ± 10.6[c]	26.3 ± 0.8[c]
LA[d]	156 ± 19[e]	38.9 ± 2.5[e]	31.6 ± 3.5[e]	162 ± 6[e]	59.6 ± 4.5	56.8 ± 8.7[c]	26.8 ± 1.6[c]
BSO	98.8 ± 4.0[f]	27.1 ± 2.9[c]	23.3 ± 4.3[c,e]	123 ± 6[c]	58.0 ± 2.1	25.2 ± 4.7[e]	31.0 ± 1.5[e]

[a]Data are means ± SE of six rats. CHOL, cholesterol; TG, triglyceride; and PL, phospholipid.
[c,e,f,g]Values not sharing a common superscript letter are significantly different at $P < 0.05$.
[b]ALA-rich fat.
[d]LA-rich fat.

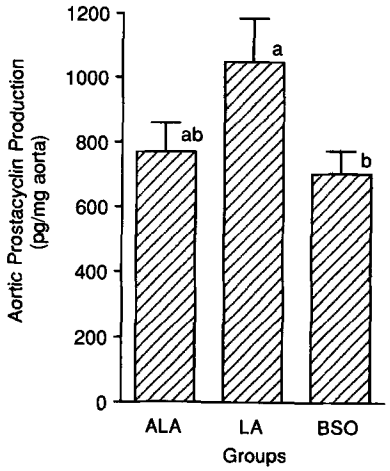

FIG. 1. Effect of ALA- and LA-rich fats and BSO on aortic prostacyclin production in rats. Prostacyclin was measured as a 6-keto-prostaglandin $F_{1\alpha}$. Data are means ± SE of six rats. Values not sharing a common letter are significantly different at $P < 0.05$.

maximum platelet aggregation induced by 5 μM adenosine diphosphate (ADP) among the three groups (32.7 ± 6.0%, 33.1 ± 4.1% and 39.0 ± 4.0% in the ALA-rich fat, LA-rich fat and BSO groups, respectively; mean ± SE of six rats).

Fatty acid compositions. Fatty acid compositions of liver PC and PE are shown in Table 4. The proportions of LA in the respective phospholipid fractions were similar among the three groups. The proportion of AA was lower when the dietary fat was BSO or ALA than when it was LA. BSO decreased AA more markedly than ALA-rich fat in liver PC, but not in PE. The same tendency was observed in plasma PC (data not shown). The proportion of EPA in both phospholipid fractions was considerably higher in rats fed ALA-rich fat than in those fed BSO; essentially no EPA was detected in rats fed the LA-rich fat. However, the proportions of 22:5n-3 and 22:6n-3 were not too different between the ALA-rich fat and the BSO groups. The proportion of LA in triglyceride and cholesteryl ester of liver was significantly lower in the BSO and ALA-rich fat groups than in the LA-rich fat group, but no difference between the BSO and ALA-rich fat groups was found (data not shown).

The fatty acid composition of epididymal adipose tissue total lipids reflected that of the dietary fat, as shown in Table 5. The proportions of LA and AA were significantly higher in the LA-rich fat group than in the ALA-rich fat or the BSO groups.

Incorporation of 20:3BSO and 20:4BSO into various lipid fractions is summarized in Table 6. Markedly higher incorporation of 20:3BSO, as compared to 20:4BSO, was seen in the phospholipid fractions of plasma and liver. In contrast, more 20:4BSO was incorporated than 20:3BSO in liver cholesteryl ester. Incorporation of these two PUFA into liver triglyceride was marginal.

Experiment 2

Fatty acid compositions of liver PC at the end of the experiment (feeding PUFA for 10 days) are shown in Table 7. The effect of PUFA on the fatty acid composition was more pronounced after consecutive administration for 10 days than after 3 days. The proportion of AA significantly decreased, and that of 20:3n-9 increased in liver PC of rats fed the fat-free diet. ALA was the most effective in decreasing the proportion of 20:3n-9, while it simultaneously decreased AA. The ability of 20:4BSO to decrease 20:3n-9 was equal to that of LA. The increase in AA in the liver PC fraction was most evident when LA was administered. When GLA or 20:4BSO was administered, the proportion of AA was similar to that of the fat-free group. ALA administration resulted in the lowest AA values. The high 20:3n-9/AA ratio in the fat-free group was apparently

TABLE 4

Fatty Acid Composition of Liver Phosphatidylcholine and Phosphatidylethanolamine[a]

Fatty acid	Phosphatidylcholine			Phosphatidylethanolamine		
	ALA[b]	LA[c]	BSO	ALA[b]	LA[c]	BSO
16:0	17.1 ± 1.0[d]	16.8 ± 0.9[d]	22.7 ± 1.3[e]	13.2 ± 1.0	13.4 ± 0.8	15.2 ± 0.9
16:1	2.1 ± 0.3[d,e]	1.6 ± 0.2[d]	2.6 ± 0.3[e]	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
18:0	18.4 ± 1.1[d]	15.0 ± 0.9[e]	13.3 ± 0.7[e]	25.5 ± 0.7	22.4 ± 1.2	22.5 ± 1.2
18:1	10.9 ± 0.4	10.1 ± 0.5	10.8 ± 0.6	7.1 ± 0.6[d,e]	8.3 ± 0.6[d]	6.4 ± 0.4[e]
18:2	17.1 ± 0.7	15.5 ± 1.7	17.4 ± 3.0	5.2 ± 0.8	5.2 ± 0.8	4.6 ± 0.4
18:3n-3	0.8 ± 0.1[d]	—	0.5 ± 0.1[e]	0.5 ± 0.1	—	0.4 ± 0.1
20:3BSO	—	—	10.8 ± 1.0[f]	—	—	7.3 ± 0.8[f]
20:3n-6	1.9 ± 0.1[d]	1.1 ± 0.2[e]	—	0.5 ± 0.0	0.5 ± 0.1	—
20:4n-6	13.1 ± 1.2[d]	32.2 ± 1.3[e]	8.7 ± 0.1[g]	14.2 ± 1.0[d]	34.0 ± 1.3[e]	14.4 ± 1.0[d]
20:4BSO	—	—	1.1 ± 0.1	—	—	0.7 ± 0.4
20:5n-3	9.1 ± 0.6[d]	—	2.9 ± 0.2[e]	13.6 ± 0.3[d]	—	6.3 ± 0.3[e]
Unknown	—	1.7 ± 0.3	—	—	1.6 ± 0.2	—
22:5n-6	—	—	—	—	3.7 ± 1.0	—
22:5n-3	3.1 ± 0.3[d]	1.4 ± 0.1[e]	2.0 ± 0.2[e]	6.0 ± 0.3[d]	2.8 ± 0.5[e]	5.7 ± 0.2[d]
22:6n-3	4.8 ± 0.4[d]	1.7 ± 0.1[e]	4.7 ± 0.3[d]	13.3 ± 1.4[d]	5.5 ± 0.4[e]	15.5 ± 0.7[d]

[a]Data are means ± SE of 6 rats. 20:3BSO, 5*c*,11*c*,14*c*-eicosatrienoic acid; 20:4BSO, 5*c*,11*c*,14*c*,17*c*-eicosatetraenoic acid.

[b]ALA-rich fat.

[c]LA-rich fat.

[d,e,g]Values not sharing a common superscript letter are significantly different at $P < 0.05$.

[f]Includes 20:3n-6.

TABLE 5

Fatty Acid Composition of Epididymal Adipose Tissue Total Lipids[a]

Fatty acid	ALA[b]	LA[c]	BSO
16:0	16.8 ± 1.3[d]	17.1 ± 1.4[d]	21.5 ± 0.7[e]
16:1	5.2 ± 0.7	5.2 ± 0.5	6.0 ± 0.4
18:0	3.4 ± 0.2[d]	2.8 ± 0.1[d]	4.1 ± 0.3[e]
18:1	30.9 ± 1.1	28.8 ± 0.7	30.6 ± 1.0
18:2	17.0 ± 1.1[d]	41.1 ± 1.7[e]	16.9 ± 1.0[d]
18:3n-3	21.4 ± 1.4[d]	—	13.5 ± 0.9[e]
20:3BSO	—	—	1.2 ± 0.1
20:4n-6	0.5 ± 0.1[d]	1.1 ± 0.1[e]	0.4 ± 0.0[d]
20:4BSO	—	—	2.2 ± 0.1
20:5n-3	0.7 ± 0.1[d]	—	0.3 ± 0.1[e]
22:5n-6	—	0.2 ± 0.0	—
22:5n-3	2.6 ± 0.3[d]	1.9 ± 0.2[d,e]	1.8 ± 0.2[e]

[a]Data are means ± SE of six rats. 20:3BSO, 5*c*,11*c*,14*c*-eicosatrienoic acid; and 20:4BSO, 5*c*,11*c*,14*c*,17*c*-eicosatetraenoic acid.

[b]ALA-rich fat.

[c]LA-rich fat.

[d,e]Values not sharing a common superscript letter are significantly different at $P < 0.05$.

TABLE 6

Incorporation of 20:3BSO and 20:4BSO into Various Lipid Fractions in Rats Fed BSO[a]

Lipid fraction	20:3BSO	20:4BSO
	(% of total fatty acid)	
Plasma PC	17.8 ± 1.1	1.3 ± 0.1
Plasma CE	6.6 ± 0.3	9.1 ± 1.1
Plasma TG	2.9 ± 0.2	3.5 ± 0.4
Liver PC	10.8 ± 1.0	1.1 ± 0.1
Liver PE	7.3 ± 0.8	0.7 ± 0.4
Liver CE	1.7 ± 0.1	9.9 ± 1.0
Liver TG	0.1 ± 0.1	0.2 ± 0.1
Adipose TL	1.2 ± 0.1	2.2 ± 0.1

[a]Data are means ± SE of six rats. PC, phosphatidylcholine; PE, phosphatidylethanolamine; CE, cholesterol ester; TG, triglyceride; and TL, total lipids.

reduced by the administration of LA. THe effects of administering GLA, ALA or 20:4BSO were similar and were less than that of LA. The same tendency was observed in the fatty acid composition of liver PE (data not shown). Various PUFA did not show a different effect on plasma and liver lipid concentrations, the production of aortic PGI_2 or platelet thromboxane A_2 (data not shown).

DISCUSSION

Our results show that BSO lowered plasma cholesterol more effectively than the fats rich in either LA or ALA. The effect of BSO can be ascribed to 20:3BSO and 20:4BSO, because the content of PUFA in the dietary fats was adjusted to be the same. A reduction in plasma triglycerides and phospholipids also was observed in rats fed BSO as compared to those fed LA-rich fat, although the efficacy was comparable to that of ALA-rich fat. The most specific effect of BSO was seen in liver triglyceride. The concentration of liver triglyceride in the BSO group was less than half that in the LA-rich and ALA-rich fat groups. The observation suggests that BSO specifically inhibits the synthesis of triglyceride and/or promotes the catabolism of triglyceride in the liver. The decrease in liver triglyceride may cause reduced synthesis and secretion of very low density lipoprotein (VLDL) into plasma (11). This may be the reason why BSO decreased plasma cholesterol concentration more efficiently than fat rich in LA or ALA. However, since the plasma triglyceride concentration in rats fed BSO was comparable to that in the ALA-rich fat group, the plasma clearance rate of VLDL triglyceride in the BSO group might be different from that in the ALA-rich fat group. Although EPA synthesized from ALA depresses the secretion of VLDL and decreases

TABLE 7

Fatty Acid Composition of Liver Phosphatidylcholine (10 days)[a]

Fatty acid	Safflower[b]	Fat-free	LA	GLA	ALA	BSO
16:0	13.8 ± 0.7[c]	16.2 ± 0.5[d,f]	16.4 ± 0.4[d]	14.7 ± 0.4[c,f]	19.5 ± 0.5[e]	16.2 ± 0.7[d,f]
16:1	2.0 ± 0.3[c]	7.7 ± 0.4[d]	5.2 ± 0.4[e,f]	6.1 ± 0.5[e]	5.3 ± 0.2[e,f]	4.3 ± 0.2[f]
18:0	21.6 ± 0.9[c]	17.0 ± 0.5[d]	19.5 ± 0.9[c,d]	20.2 ± 1.0[c]	19.6 ± 0.5[c,d]	20.6 ± 0.8[c]
18:1	9.8 ± 0.7[c]	20.8 ± 0.4[d]	16.5 ± 0.6[e]	17.2 ± 0.5[e]	19.6 ± 0.5[d,f]	18.0 ± 0.8[e,f]
18:2	7.6 ± 0.4[c]	4.9 ± 0.3[d]	6.9 ± 0.7[c]	2.9 ± 0.2[e]	3.3 ± 0.1[e]	3.2 ± 0.1[e]
18:3n-6	0.0 ± 0.0[c]	0.7 ± 0.1[c]	0.2 ± 0.1[c]	12.0 ± 1.0[d]	0.2 ± 0.0[c]	0.3 ± 0.0[c]
20:3n-9	0.5 ± 0.1[c]	19.2 ± 0.5[d]	11.5 ± 0.3[e]	13.9 ± 0.8[f]	9.7 ± 0.6[g]	12.0 ± 0.7[e]
20:3n-6	1.5 ± 0.1[c]	0.0 ± 0.0[d]	3.4 ± 0.1[e]	1.1 ± 0.7[c,d]	0.4 ± 0.3[d]	0.5 ± 0.3[c,d]
20:4n-6	35.1 ± 1.4[c]	6.1 ± 0.7[d,f]	14.2 ± 0.5[e]	7.3 ± 0.3[d]	4.6 ± 0.4[f]	6.7 ± 0.4[d]
20:4BSO	—	—	—	—	—	6.0 ± 0.5
20:5n-3	0.1 ± 0.0[c]	0.3 ± 0.0[c]	0.2 ± 0.0[c]	0.3 ± 0.0[c]	3.4 ± 0.2[d]	1.0 ± 0.1[e]
22:5n-6	5.5 ± 0.5[c]	1.5 ± 0.1[d]	2.8 ± 0.3[e]	1.7 ± 0.1[d]	0.4 ± 0.0[f]	1.8 ± 0.2[d]
22:5n-3	0.3 ± 0.1[c,e]	0.4 ± 0.1[c,e]	0.3 ± 0.0[c]	0.2 ± 0.0[c]	0.7 ± 0.1[d]	0.5 ± 0.0[e]
22:6n-3	1.1 ± 0.1[c]	1.4 ± 0.1[c]	1.4 ± 0.1[c]	1.5 ± 0.0[c]	12.6 ± 0.3[d]	7.3 ± 0.5[e]
20:3n-9/20:4n-6	0.01 ± 0.00[c]	3.3 ± 0.4[d]	0.82 ± 0.04[e]	1.9 ± 0.2[f]	2.2 ± 0.2[f]	1.8 ± 0.1[f]

[a]Data are means ± SE of 5-6 rats. 20:4BSO, 5*c*,11*c*,14*c*,17*c*-eicosatetraenoic acid.

[b]Safflower oil-supplemented diet.

[c-f]Values not sharing a common superscript letter are significantly different at $P < 0.05$.

the plasma concentration of triglyceride and cholesterol in rats, it does not decrease the concentration of liver triglyceride significantly (12). These observations point towards a specific effect of BSO on hepatic triglyceride metabolism.

The hypocholesterolemic effect of LA, the most ubiquitous n-6 PUFA, is widely accepted, although the precise mechanism is still under debate (13). It has been reported that excessive intake of LA may result in various side-effects, including promotion of carcinogenesis, increased lithogenecity, acceleration of cell aging, immunodepression, and lowering of HDL cholesterol (14,15). These effects can be mostly, but not entirely, explained by the overproduction and imbalance of specific prostaglandin(s). Therefore, PUFA having a cholesterol-lowering effect and not serving as a substrate for 2-series prostaglandins may be more useful. EPA contained in fish oil has been reported to exert a hypotriglyceridemic and hypocholesterolemic effect, and it interferes with the production of the 2-series prostaglandins in rats (16). However, fish oil is extremely sensitive to peroxidation and is not a good dietary source because of its unpleasant odor. BSO lowered plasma cholesterol without increasing prostaglandin synthesis; therefore, it can be a useful hypolipidemic fat compared to the usual n-3 PUFA, such as EPA and ALA.

The proportion of AA in plasma and liver PC decreased in rats fed ALA-rich fat or BSO, suggesting interference with desaturation and elongation of LA to AA. The reduction of AA in liver and plasma PC of the BSO group was more pronounced than in the ALA-rich group. The reduction of tissue AA may be related to the decrease in aortic PGI_2 synthesis. Since BSO and ALA-rich fat decreased PGI_2 synthesis to the same extent as compared to LA-rich fat, it is quite apparent that BSO has an effect similar to that of ALA.

Incorporation of 20:3BSO and 20:4BSO into various lipid fractions was specific; more 20:3BSO than 20:4BSO was incorporated into liver PC, PE and plasma PC, whereas the opposite was true for incorporation into liver cholesteryl esters and adipose tissue triglycerides. The proportions of these fatty acids in plasma cholesteryl ester and triglyceride were comparable to those of dietary BSO. These fatty acids were essentially not incorporated into liver triglyceride. The metabolic consequence of such a tissue and lipid-specific incorporation of these anomalous PUFA is not obvious at present. However, it is plausible that the deposition of a considerable amount of 20:3BSO into membrane phospholipids causes an alteration in membrane properties and function.

The ability of 20:4BSO to act as an essential fatty acid was assessed by the reduction in the ratio of 20:3n-9/AA in liver PC, which was comparable to that of GLA or ALA, and less than that of LA. ALA most effectively decreased the proportion of both AA and 20:3n-9, suggesting a greater depression by ALA of the desaturation and elongation of oleic acid, as well as LA (17). Although 20:4BSO, another n-3 PUFA, also tended to decrease AA and 20:3n-9, the effect was less than that of ALA. In contrast, BSO was more effective in decreasing AA than ALA-rich fat in the liver PC fraction in Exp. 1. The difference can be explained by the difference in the essential fatty acid status or in the composition of fat given between these two experiments. In Exp. 1, dietary fat contained the mixture of LA, ALA, 20:3BSO and 20:4BSO, in contrast to ethyl 20:4BSO in Exp. 2.

The improvement of skin symptoms due to administration of PUFA was not evident in these experiments. It has been reported that n-3 PUFA, when compared to LA, are less effective in improving skin symptoms resulting from essential fatty acid deficiency (18,19).

The results in Exp. 1 showed that 20:3BSO and/or 20:4BSO contained in BSO have some specific effects on lipid metabolism in rats. It also was suggested that 20:4BSO may function as an essential fatty acid, as would ALA and GLA, as judged by the decrease in the ratio of 20:3n-9/AA. Therefore, BSO may become useful as a beneficial dietary PUFA to prevent lipid disorders.

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Phospholipid Fatty Acyl Distribution of Three Fungi Indicates Positional Specificity for n-6 *vs.* n-3 Fatty Acids

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Phospholipids of the fungi *Conidobolus nanodes, Entomophthora exitalis* and *Saprolegnia parasitica* were extracted and analyzed. The phospholipid content was the same (2.4%) for the three species and was independent of the total lipid content. Phospholipase A_2 degradation of individual phospholipid classes showed an asymmetrical distribution of polyunsaturated fatty acids (PUFA) between the two fatty acyl positions of glycerol. There was a predominance of n-6 PUFA at position 2 and a predominance of n-3 PUFA at position 1. With *C. nanodes* and *E. exitalis*, 20:5n-3 is derived from 18:3n-3 and is located predominantly at position 1. In *S. parasitica* 20:5n-3 is synthesized from 18:3n-6 *via* 20:4n-6 and is located predominantly at position 2. It is suggested that the asymmetrical distribution of PUFA between positions 1 and 2 of glycerol points towards different sites of synthesis of the two classes of PUFA, and that cross-over between PUFA of the different types is prevented by the *sn*-1 or *sn*-2 positional specificity of the desaturases.
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Phospholipids are highly versatile membrane components that can, for example, regulate changes in membrane fluidity in response to changes in growth temperature, oxygen deprivation or salinity (1). When working with the fungus *Entomophthora exitalis,* we observed changes in phospholipid composition with respect to the proportion of the phospholipid classes that were present and to the degree of fatty acyl unsaturation in response to changing growth temperatures (2). The phospholipid fraction of this organism, compared to the neutral, glycolipid and sphingolipid fractions, contains the highest proportion of polyunsaturated fatty acids (PUFA) (2–4). This difference in distribution of PUFA between lipid classes could either reflect a functional necessity of phospholipids within the various cell membranes, or indicate that phospholipids are the sites of PUFA biosynthesis, or both. Phospholipids have actually been shown to serve as substrates for fatty acyl desaturation in eukaryotic organisms, including yeasts (5,6) and plants where, for example, in barley *(Hordeum vulgare)* oleate is desaturated to linoleate and then to linolenate while being a constituent of phosphatidylcholine (PC) (7).

The three fungi selected for this work were part of a survey of PUFA-producing fungi (2,3). All displayed a predominance of PUFA in the phospholipid fractions as described above. All three fungi are members of the lower fungi or phycomycetes (8). *Saprolegnia parasitica* is an Oomycetes and has been reported as being able to synthesize eicosapentaenoic acid (EPA) from arachidonic acid (AA) (9,10), *i.e.,* it possesses a Δ17 desaturase. *Entomophthora exitalis* and *Conidiobolus nanodes* are both Zygomycetes (8) and have been reported (11–13) as producing long-chain PUFA of both the n-6 type (*e.g.,* AA) and the n-3 type [*i.e.,* EPA and docosahexaenoic acid (DHA)]. We therefore felt that the analysis of the positional distribution of the fatty acyl groups in glycerol positions 1 and 2 of the phospholipids of these fungi may help in a better understanding of PUFA biosynthesis in fungi in general.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetic acid disodium salt; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; MES, 4-morpholinoethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid.

MATERIALS AND METHODS

Micro-organisms and growth. The strains of fungi examined were *Conidiobolus nanodes* (IMI 92299), *Entomophthora exitalis* (NRRL 3742) and *Saprolegnia parasitica* (ATCC 22284). The fungi were cultivated in semi-defined nitrogen-limiting growth media which combined (g/L): Glucose, 30; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 1.5; yeast extract (Oxoid) 1.5; $CaCl_2 \cdot 2H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.008; $ZnSO_4 \cdot 7H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.0001; $Co(NO_3)_2 \cdot 6H_2O$, 0.0001; $MnSO_4 \cdot 5H_2O$, 0.0001 and diammonium tartrate, 3.3. The fungi were grown for 72 h in 800 mL media contained in 1-L vortex-aerated reagent bottles. *C. nanodes* and *E. exitalis* were grown at 30°C and *S. parasitica* was grown at 24°C. Lipids were extracted with chloroform/methanol (2:1, vol/vol), fractionated using a column of silicic acid, and fatty acids were analyzed as methyl esters by gas chromatography as previously described (3).

Thin-layer chromatographic analysis of phospholipids (3). Separation of phospholipids was achieved on 0.25 mm Silica Gel G plates (Anachem, Luton, Bedshire, U.K.) (20 × 20 cm) using chloroform/methanol/water/acetic acid (65:43:3:1, by vol) for development. Spots were visualized by staining with 2′,7′-dichlorofluorescein (0.1% w/vol in ethanol) and viewed under UV light. Phospholipids were identified by comparison with authentic standards (Sigma Chemical Co., St. Louis, MO). The appropriate regions of the plate were scraped off, and phospholipids were extracted with 50 mL of chloroform/methanol (1:1, vol/vol). Silica Gel G was removed by filtration and was washed with 50 mL chloroform/methanol (1:1, vol/vol). The organic phases were pooled and the solvent was removed by rotary evaporation.

Phospholipase A_2 degradation of phospholipids (9). Phospholipids (5–10 mg) were dissolved in diethyl ether (5 mL) containing hexadecyltrimethylammonium bromide (20 mg); 10 mM 4-morpholinoethenesulfonic acid (MES) buffer, pH 7.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 100 units phospholipase A_2 (porcine pancreas; Sigma), was added (1 mL) and the mixture was shaken vigorously for 3 h at 37°C. The aqueous phase was removed and washed with diethyl ether (3 × 50 mL). The ether washings were combined with the ether layer, the solvent was evaporated and the residue (the free fatty acids) was redissolved in a minimal volume of diethyl ether, weighed after evaporation of the solvent, and stored

at −20°C. The aqueous layer was mixed with ethanol (20 mL), the solvent was removed by rotary evaporation and the residue of lysophospholipids was redissolved in a minimal volume of diethyl ether and stored at −20°C.

RESULTS

The phospholipid contents of the three fungi examined were similar at 2.5% w/w of biomass (Table 1), and appeared to be independent of the total lipid content. In each case, the main component of the extracted lipid was triacylglycerol (data not given, see ref. 3).

The distribution of fatty acids between the *sn*-1 and *sn*-2 positions of the phospholipids was examined for each fungus (Tables 2, 3 and 4). With one exception, position

TABLE 1

Phospholipid Composition of Fungi[a]

Fungus	Lipid content (%, w/w)	Phospholipid (%, w/w)	Relative proportion of phospholipid classes (%, w/w)			
			PC	PE	PI[a]	PS[a]
C. nanodes	25.3	2.4	35.0	38.9	26.1	ND[b]
E. exitalis	24.6	2.3	65.0	17.8	14.7	ND
S. parasitica	10.6	2.6	36.1	28.2	ND	35.7

[a]PI, phosphatidylinositol; PS, phosphatidylserine.
[b]ND, not detected.

TABLE 2

Positional Distribution of Fatty Acids in Phospholipids Isolated from *C. nanodes*

Fatty acid[a]	Relative % (w/w) of fatty acyl groups					
	PC		PE		PI	
	1	2	1	2	1	2
14:0	6.8	5.9	11.6	3.2	4.0	1.1
14:1	7.3	2.0	6.5	8.3	1.7	0.6
14:2	3.6	1.4	0.1	0.1	2.0	0.1
16:0	25.1	13.6	28.2	11.5	32.8	5.8
16:1	8.6	7.9	9.1	8.8	6.3	10.2
16:2	—	20.3	0.1	0.1	0.3	2.7
18:0	2.0	2.9	1.7	1.9	0.8	1.4
18:1	31.7	34.8	32.2	30.9	39.3	37.2
18:2	3.7	1.7	1.6	1.9	3.5	3.1
18:3n-3	0.1	0.1	0.1	0.1	0.1	0.1
18:3n-6	0.1	0.1	0.1	0.1	0.1	0.5
20:0	0.1	—	—	4.6	—	0.1
20:1	0.1	0.3	0.6	0.1	0.1	3.4
20:2	—	0.8	—	—	1.5	1.7
20:3n-6	0.6	1.2	0.1	1.2	0.6	3.8
20.4n-6	1.3	6.2	1.8	27.2	3.9	27.9
20:5n-3	2.2	0.6	1.1	—	1.2	—
22:5n-3	—	—	0.1	—	0.2	—
22:6n-3	6.4	0.2	5.0	—	1.6	0.3
PUFA (total)	14.4	32.6	10.1	30.7	18.0	40.2
PUFA (n-6)	2.0	7.5	2.0	28.2	4.6	32.2
PUFA (n-3)	8.7	0.9	6.3	—	1.9	0.4

[a]Expressed as percentages (w/w) of total fatty acids present.

TABLE 3

Positional Distribution of Fatty Acids in Phospholipids Isolated from *E. exitalis*

Fatty acid[a]	Relative % (w/w) of fatty acyl groups					
	PC		PE		PI	
	1	2	1	2	1	2
14:0	1.9	1.8	2.2	3.5	4.9	0.9
14:1	0.1	0.2	0.3	3.1	0.6	0.1
14:2	0.3	0.1	0.6	0.7	0.1	0.1
16:0	10.7	7.3	13.9	18.1	20.2	4.8
16:1	7.0	15.4	6.5	10.8	8.0	7.0
16:2	1.0	3.0	0.3	3.9	0.5	0.3
18:0	0.1	2.1	1.0	5.1	0.4	3.1
18:1	51.8	51.4	53.1	33.9	55.6	40.2
18.2	4.8	5.2	6.0	1.7	2.5	4.2
18:3n-6	0.4	0.1	0.1	0.1	0.1	0.3
18:3n-3	1.4	0.5	0.1	0.1	0.5	0.6
20:0	0.2	2.9	1.1	2.4	0.4	2.3
20:1	0.3	0.1	0.1	1.1	0.6	0.7
20:2	0.6	—	0.1	0.2	0.8	0.3
20:3n-6	1.4	0.9	0.4	2.1	0.4	6.1
20:4n-6	2.5	9.0	6.0	13.1	1.9	28.8
20:5n-3	0.8	—	1.3	0.1	0.6	0.1
22:5n-3	0.1	—	0.3	—	—	—
22:6n-3	14.6	—	6.6	—	1.5	0.1
PUFA (total)	27.9	18.8	21.8	22.0	10.4	40.9
PUFA (n-6)	4.3	10.0	6.5	15.3	2.4	35.2
PUFA (n-3)	16.9	0.5	8.3	0.2	2.6	0.8

[a]Expressed as percentage (w/w) of total fatty acids present.

TABLE 4

Positional Distribution of Fatty Acids in Phospholipids Isolated from *S. parasitica* (ATCC 22284)

Fatty acid[a]	Relative % (w/w) of fatty acyl groups					
	PC		PE		PI	
	1	2	1	2	1	2
14:0	2.1	2.7	4.7	2.1	4.6	3.8
14:1	2.0	2.2	1.4	1.1	6.3	2.9
14:2	1.0	0.8	1.3	1.2	3.6	0.5
16:0	21.0	13.2	21.9	13.8	17.5	15.8
16:1	16.3	7.5	9.0	6.7	13.5	7.6
16:2	2.1	1.5	2.6	3.5	2.5	1.6
18:0	4.8	7.5	5.2	6.3	3.6	2.2
18:1	39.0	18.7	34.6	30.0	40.3	16.3
18:2	4.5	5.2	13.5	4.8	2.2	6.6
18.3n-3	—	—	—	—	—	—
18:3n-6	0.2	11.4	1.0	10.5	2.2	11.0
20:0	1.4	0.9	0.1	0.1	0.1	1.2
20:1	1.8	1.8	0.1	0.1	0.1	0.1
20:2	0.1	0.3	0.2	0.1	0.1	0.1
20:3n-6	0.1	0.4	0.9	1.0	0.1	0.3
20:4n-6	2.0	13.8	3.2	11.0	1.1	12.0
20:5n-3	1.6	12.1	0.3	7.7	2.2	18.0
PUFA (total)	11.6	45.5	23.0	39.8	14.0	50.1
PUFA (n-6)	2.3	25.6	5.1	22.5	3.3	41.3
PUFA (n-3)	1.6	12.1	0.3	7.7	2.2	18.0

[a]Expressed as percentage (w/w) of total fatty acids present.

2 contained a greater proportion of PUFA than position 1, the exception being PC isolated from *E. exitalis*.

A further pattern emerges when the fatty acid profiles are expressed as n-3 and n-6 PUFA at each position. In the fungi *C. nanodes* (Table 2) and *E. exitalis* (Table 3), n-3 PUFA predominate at position 1 and n-6 PUFA at position 2. In *S. parasitica*, the only n-3 PUFA evident is EPA (Table 4), which is fund preferentially at position 2 along with the n-6 PUFA, AA. It has been shown previously that *S. parasitica* belongs to a group of organisms that can synthesize EPA from AA (9,10), *i.e.*, they possess a Δ17 desaturase. Accordingly, if the two groups of PUFA are reclassified, either as derivatives of gamma-linolenic acid (GLA) (*i.e.*, n-6 PUFA) or α-linolenic acid (*i.e.*, n-3 PUFA), a consistent pattern of fatty acyl distribution can now be discerned for all three fungi—position *sn*-1 contains predominantly PUFA derived from α-linolenic acid, and position *sn*-2 contains predominantly PUFA derived from GLA.

DISCUSSION

Predominance of PUFA at position 2 of phospholipids has been reported previously to the fungus *Phycomyces blakesleeanus* (14), which is classed as a lower fungus and produces only n-6 PUFA, and also in rat (15), rainbow trout (16) and mosses (9). No evidence has been presented to explain such a distribution resulting from *de novo* phospholipid biosynthesis, *i.e.*, the transfer of CDPcholine to specific 1,2-diacylglycerol species with a preference of n-3 in position 1 and n-6 in position 2. Neither has asymmetrical distribution of different PUFA classes been reported in a 1,2-diacylglycerol.

Phospholipids in eukaryotic systems other than animals are accepted as the most likely form in which fatty acids exist for desaturation (5–7). The results obtained here could be viewed as evidence for separate sites of n-3 and n-6 PUFA synthesis on phospholipid molecules (positions 1 and 2, respectively). This theory is supported by comparing the positional distribution of fatty acids of the fungi *C. nanodes* and *E. exitalis* with the fungus *S. parasitica*. In the former fungi, EPA is derived from α-linolenic acid, 18:3n-3, and is located predominantly at position 1. In the latter fungus, EPA is derived from GLA 18:3n-6, and is located predominantly at position 2. The change in positional distribution in response to the changed mode of EPA synthesis would support the theory of n-3 and n-6 PUFA biosynthesis at positions 1 and 2, respectively. By extension of this argument, as both 18:3 isomers will be derived from 18:2 (*c*9,*c*12), the Δ6 desaturase must act primarily on the acyl group at position 2 and the Δ15 desaturase must act on the acyl group at position 1. This prediction has been confirmed in plants by work with borage *(Borage officinalis)* preparations which has revealed that, although linoleate is present at both positions of PC, only linoleate at position 2 acts as a substrate for the Δ6 desaturase to give GLA (17). We have recently made a similar observation for the formation of GLA in *Mucor circinelloides*, except that phosphatidylethanolamine (PE) is used as the acyl carrying phospholipid (Kendrick, A.J., and Ratledge, C., unpublished work).

In *C. nanodes* and *E. exitalis*, the PUFA which predominate are the n-6 type (3). Of the three phospholipid types present, PC had the lowest proportion of n-6 PUFA. Further work with borage preparations (18) has shown that, although other phospholipid types are substrates for desaturation, only PC is actively turned over and is involved in the supply of fatty acids for other cellular lipids (14). If PC is similarly turned over in *C. nanodes* and *E. exitalis* then it could be predicted that the n-6 PUFA content of this phospholipid would be lower than that of the other phospholipid types and, indeed, this has been shown to be the case in this work.

The present observations allow us to speculate that n-6 and n-3 PUFA diverge from their common precursor of 18:2 not only by there being two distinct desaturases (the Δ6 and the Δ15, respectively), but by these enzymes acting at different sites on the phospholipid carrying the 18:2 residues. Thus, one can understand how each series of n-6 and n-3 PUFA is created without spurious introduction of double bonds into the wrong position. The two series of PUFA are kept distinct and separate by virtue of their differential attachments to the phospholipid carrier. Cross-over from n-6 to n-3 thus remains minimized. The only exception comes with *Saprolegnia parasitica* for the final conversion of 20:4n-6 (AA) to 20:5n-3 (EPA), which occurs with a Δ17 desaturase acting upon AA at the *sn*-2 position. The presence of this enzyme in this fungus has already been described by Gellerman and Schlenk (10) and, more recently, in *Mortierella alpina* (19). (The stereospecific distribution of PUFA on the phospholipids of the latter mold has not been carried out to verify if it fits in with the pattern observed here.) In the other two fungi studied here there is no evidence for cross-over, and EPA is seemingly synthesized from 18:3n-3, probably *via* 20:3n-3 and 20:4n-3, which remain attached to the *sn*-1 position in the phospholipids.

The proof of this hypothesis will need to await the isolation and detailed study of the appropriate desaturases. Nevertheless the analyses recorded here offer an explanation for the origin of n-6 and n-3 PUFA in the same fungal organism, and possibly in those plants that produce both n-6 and n-3 PUFA. It would clearly be of interest to see whether this hypothesis was of general applicability in fungi and in plants. An examination of the stereospecific distribution of PUFA in ascomycete and basodiomycete fungi (the "fungi imperfect" and "higher fungi"), which produce only n-3 PUFA, should be extremely revealing. If our hypothesis holds, such fungi would be expected to have 18:3n-3, and any other n-3 PUFA, located only at the *sn*-1 position of the appropriate phospholipid involved in the desaturase reactions, as this would be the site of action of the Δ15 desaturase.

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Inhibitory Effect of Curcumin on Fatty Acid Desaturation in *Mortierella alpina* 1S-4 and Rat Liver Microsomes

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An extract of rhizomes of *Curcuma longta* L. (turmeric) inhibited the desaturation of dihomo-γ-linolenic acid (DGLA) in the arachidonic acid (AA) producing fungus *Mortierella alpina* 1S-4. The factor responsible for this phenomenon was isolated and identified as curcumin (diferuloyl methane). Mycelial DGLA levels increased about two-fold (22.3 mg/g dry weight) with a concomitant decrease in AA levels when the fungus was cultivated with curcumin. The 50% inhibitory concentration against Δ5 desaturase was 27.2 μM. Curcumin also inhibited rat liver microsomal Δ5 and Δ6 desaturases.
Lipids 27, 509–512 (1992).

Polyunsaturated fatty acids (PUFA), especially those of the C_{20} series, such as dihomo-γ-linolenic acid (8,11,14-*cis,cis,cis*-eicosatrienoic acid, DGLA), arachidonic acid (5,8,11,14-*cis,cis,cis,cis*-eicosatetraenoic acid, AA) and 5,8,11,14,17-*cis,cis,cis,cis,cis*-eicosapentaenoic acid, are not only important precursors of prostaglandins, but they also regulate membrane fluidity as primary components of biological membranes and affect various physiological activities. In higher animals, PUFA of the ω6 and ω3 series cannot be synthesized unless linoleic acid (ω6) or α-linolenic acid (ω3) is supplied in the diet. Thus, compounds that regulate the metabolism of these PUFA are of profound interest, as they may not only serve as important means of clarifying the physiological significance of PUFA biosynthesis, but they also may be useful as new types of drugs.

We have reported previously (1,2) that sesamin and other lignan compounds found in sesame seeds inhibit the conversion of DGLA to AA (Δ5 desaturation). In the present study we tested several extracts of crude drugs and spices for their effects on the fatty acid composition of the AA producing fungus, *Mortierella alpina* 1S-4. We found that the DGLA/AA ratio in the fungus increased significantly when grown in a medium supplemented with an ethanol-extract of turmeric. In this paper we report the isolation and characterization of the inhibitory constituent responsible for this phenomenon and its effect on the desaturation system of this fungus, as well as on rat liver microsomal desaturases.

MATERIALS AND METHODS

Chemicals. Curcumin was purchased from Wako Pure Chemicals (Osaka, Japan). Turmeric, other spices and crude drugs were obtained from a local market. Radiolabeled fatty acids were obtained from Amersham International (Buckinghamshire, U.K.). All other reagents were of analytical grade.

Microorganism and cultivation. Mortierella alpina 1S-4 (3,4) was inoculated as a spore suspension into a 10-mL flask containing 2 mL of medium A (2% glucose and 1% yeast extract, pH 6.0) or medium B (4% glucose and 1% yeast extract, pH 6.0) and then incubated at 28°C with reciprocal shaking at 120 strokes/min, unless otherwise noted. When crude drugs and spices were tested for their effects on the fatty acid composition of the fungus, 0.5 g of each test substance was first extracted twice with 5 mL of ethanol. The extract was evaporated to dryness and then redissolved in a small volume of ethanol. The extracts were mixed with sterile medium A at concentrations of 0.1–1% (w/vol) when the fungus was inoculated and grown as described above. The ethanol concentrations were usually less than 5%.

Fatty acid analysis. Mycelial cells were harvested by suction filtration, washed with distilled water and dried at 100°C overnight for subsequent fatty acid analysis by gas-liquid chromatography (GLC) after transmethylation with methanolic HCl as detailed elsewhere (3,5).

Cell-free extract. Washed mycelia (*ca.* 7 g) of *M. alpina* 1S-4 were suspended in 1 mL of 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol and ground in an ice-cooled mortar with 2 g of sea sand for 50 min. One mL of buffer was added 10, 20, 30 and 40 min later. After centrifugation (10,000 × *g*, 4°C, 30 min), the supernatant was used as the enzyme solution.

Preparation of rat liver microsomes. Microsomes were prepared from male Wistar rats maintained on a pellet diet for 8 wk as described previously (2).

Assay of desaturase activities. The cell-free extract of *M. alpina* 1S-4 was assayed for Δ5 desaturase activity by using radiolabeled DGLA as previously described (2). Rat liver microsomal Δ5, Δ6 and Δ9 desaturase activities were measured by the method of Svensson (6) using radiolabelled DGLA, linoleic acid and stearic acid, respectively, as substrates.

Other methods. Protein concentrations were determined by the method of Bradford (7). Mass spectra were recorded on a Hitachi (Tokyo, Japan) M-80B at 70 eV. ^1H nuclear magnetic resonance (NMR) spectra were measured with Nicolet (Fermont, CA) NT-360 using CD_3OD as solvent and tetramethylsilane as internal standard.

RESULTS

Screening of Δ5 desaturation inhibitors. The effects of 62 crude drugs and 67 spices on the fatty acid profile of *M. alpina* 1S-4 were tested. The effects of some crude drugs and spices (which are commonly known and easily obtainable in Japan) on DGLA and AA production of the fungus are given in Table 1. Considering the DGLA/AA ratios of the fungus grown in the presence of the extracts as an index for the estimation of Δ5 desaturation inhibitory activity, we found that the DGLA/AA ratio of the fungus cultured with turmeric extract was 0.43. This

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Abbreviations: AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; FA, fatty acid; GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

TABLE 1

Effects of Extracts of Spices and Crude Drugs on the DGLA and AA Production of *M. alpina* 1S-4[a]

Spice	Growth (mg/mL)	FA (mg/mL) DGLA	FA (mg/mL) AA	DGLA/AA ratio
Pepper	11.4	0.38	1.48	0.26
Cinnamon	10.5	0.25	1.07	0.23
Parsley	8.4	0.10	0.35	0.29
Turmeric	9.1	0.17	0.40	0.43
Nutmeg	10.4	0.19	0.77	0.25
Tarragon	9.4	0.14	0.52	0.27
Marjoram	10.1	0.19	0.74	0.26
Coriander	9.8	0.20	0.87	0.23
Star anise	9.2	0.16	0.73	0.22
Ajowan	9.1	0.13	0.65	0.20
Mustard	9.0	0.12	0.48	0.25
Rosemary	7.1	0.08	0.40	0.20
Cumin	9.9	0.04	0.14	0.29
Thyme	10.7	0.29	1.24	0.23
Cardamom	9.9	0.17	0.73	0.23
Tochuu[b]	9.5	0.11	0.38	0.29
Nyotei[c]	7.7	0.09	0.33	0.27
Ireisen[d]	11.3	0.18	0.67	0.27
Bouhuu[e]	8.6	0.07	0.24	0.29
Goboushi[f]	11.4	0.16	0.59	0.27
Control	11.9	0.25	1.20	0.21

[a]*M. alpina* 1S-4 was grown in medium A supplemented with the extracts of the materials given in the Table. The concentrations of the extracts added were 0.1% (w/vol), and the other conditions were as described in Materials and Methods. All values are means of two determinations.

[b]Bark of *Eucommia ulmoides* Oliver.

[c]Fruit of *Liguotrum japonicum* Thunb.

[d]Root of *Clematis terniflora* DC.

[e]Root of *Seseli libanotis* Koch var. *daucifolia* DC.

[f]Fruit of *Arctium lappa* L.

TABLE 2

Effect of an Ethanol Extract of Turmeric on the Fatty Acid Profiles of *M. alpina* 1S-4[a]

Volume added (μL)[b]	Fatty acid composition (wt%) 0	5	10	20	50
16:0	13.9	20.4	18.8	22.0	25.2
18:0	8.2	8.6	8.5	7.6	7.5
18:1	7.9	15.7	16.4	20.4	25.0
18:2	6.5	8.5	8.2	9.3	9.3
18:3ω6	4.0	2.8	3.3	3.2	3.4
DGLA	4.3	6.2	6.2	7.2	10.5
AA	55.2	37.8	38.6	30.3	19.1
DGLA (mg/g)	16.9	22.0	25.7	26.5	28.7
AA (mg/g)	217.3	133.9	160.2	111.5	52.2
Total FA (mg/g)	393.7	354.2	415.1	368.0	273.3
Growth (mg/mL)	17.6	19.9	17.4	17.6	12.1
DGLA/AA ratio	0.08	0.16	0.16	0.24	0.55

[a]*M. alpina* 1S-4 was grown in medium B supplemented with the turmeric extract at 28°C for 7 d. All values are means of two determinations.

[b]The ethanol extract obtained from 0.5 g of turmeric was resuspended in 4 mL ethanol, then the indicated volumes were added to 2 mL of sterile medium A.

was about double that of the control (0.21). However, those cultured with the other extracts (including those not shown in Table 1) ranged from 0.20 to 0.29. The extracts which resulted in DGLA/AA ratio higher than 0.27 (or 1.3-fold that the control) were tested again as to their dose-dependent effects on the DGLA/AA ratio, but none of them except for turmeric extract was found to be positive (data not shown). As shown in Table 2, the presence of turmeric extract of up to 20 μL in the culture medium did not affect fungal growth, but some inhibitory effect was observed with 50 μL. The levels of palmitic acid, oleic acid and DGLA appeared to increase to some extent, whereas those of AA decreased markedly. Mycelial DGLA concentration reached 28.7 mg/g dry mycelia, a value approximately 1.7 times that of the control (no supplement).

Isolation and identification of the inhibitory component. In order to characterize the active compound(s) responsible for this inhibition, we eluted the ethanol extract through silica gel with successive solvent systems of *n*-hexane/ethyl acetate (95:5 and 90:10, vol/vol) and methanol. Based on the elution profile at 254 nm, the extract was separated into eight fractions. The inhibitory effects of each fraction were monitored by growing the fungus in medium B supplemented with each fraction (0.01–0.1% w/vol culture broth), followed by analysis of the fatty acid profile. The fungus grown in the presence of the methanol-luted fraction accumulated about twice as much DGLA (27.9 mg/g dry mycelia) than did controls (14.7 mg/g dry mycelia). DGLA concentrations were about 12–14 mg/g dry mycelia in fungi grown with the other fractions. This fraction was separated into several bands in reverse phase thin-layer chromatography (TLC, $KC_{18}F$; Whatman, Fairfield, NJ) using 70% methanol as the developing solvent. Therefore, this fraction was subfractionated on a reverse phase column packed with octadecylsilane-bonded silica gel (Nakarai Chemicals, Kyoto, Japan) using various concentrations of aqueous methanol as eluent. Seven subfractions were obtained which were separately tested for their effects on the fatty acid profile of the fungus as described above. One fraction, comprised of a yellow pigment, showed a similar effect to that found in the crude extract. The DGLA/AA ratio of the fungus grown with this fraction was 0.26, and that of the control was 0.13. On the other hand, the DGLA/AA ratios of fungi grown with the other fractions were 0.12–0.16. The yellow fraction was homogeneous in reverse phase TLC as judged by UV detection (254 nm). Mass spectra showed the molecular ion at *m/z* 368 (relative intensity 27%); the other major fragmentation ion peaks were 350 (28), 338 (3), 320 (5), 272 (9), 244 (6), 232 (12), 217 (17), 203 (5), 190 (42), 177 (100), 161 (8), 150 (26), 145 (34), 137 (50), 131 (27), 117 (22), 103 (15), 89 (27), 77 (23), 65 (13), 51 (13) and 39 (8). ^1H NMR showed signals (δ, ppm) at 3.91 (*s*, 6H, CH_3), 5.96 (*s*, 1H, C=C), 6.62 (*d*, 2H, C=C), 6.81 (*d*, 2H, Ar), 7.12 (*d*, 2H, Ar), 7.21 (*s*, 2H, Ar) and 7.57 (*d*, 2H, C=C). These data agree well with those of authentic standard and they correspond to those of the enol form of curcumin, the structure of the keto form of which is shown in Figure 1.

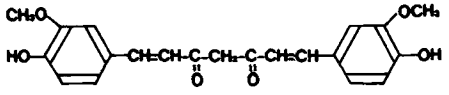

FIG. 1. Chemical structure of curcumin.

Effects of curcumin on DGLA production. Commercially obtained curcumin was dissolved in ethanol and added to sterilized medium B on which *M. alpina* 1S-4 was grown at 28°C for one week. As shown in Figure 2, the production of DGLA increased dose-dependently. The DGLA production rate was about 0.42 mg/mL culture broth per 0.1 mg/mL of curcumin. A higher concentration of curcumin inhibited fungal growth (data not shown).

Effect of curcumin on the conversion of DGLA to AA in vitro. The effect of curcumin on the conversion of DGLA to AA was investigated *in vitro* using a cell-free extract of the fungus. As shown in Figure 3a, increasing concentrations of curcumin markedly reduced the conversion of DGLA to AA. The 50% inhibitory concentration against Δ5 desaturase was 27.2 μM. On the other hand, the inhibition against Δ6 desaturase was less than 10% (data not shown). This result is consistent with the observation that the mycelial DGLA level of the fungus increased with a concomitant decrease in AA on growth in the presence of curcumin.

Inhibition effect in rat liver microsomes. Since curcumin inhibited Δ5 desaturation in the fungus, we further tested its effect on the desaturation systems of animals using rat liver microsomes (Fig. 3b). Unlike sesamin, which inhibits Δ5 desaturation in a specific manner (2), curcumin not only inhibited Δ5 desaturation, but the conversion of linoleic acid to γ-linolenic acid, *i.e.*, Δ6 desaturation. In the presence of 80 μM curcumin, the inhibition of Δ5 and Δ6 desaturation was 49% and 18%, respectively.

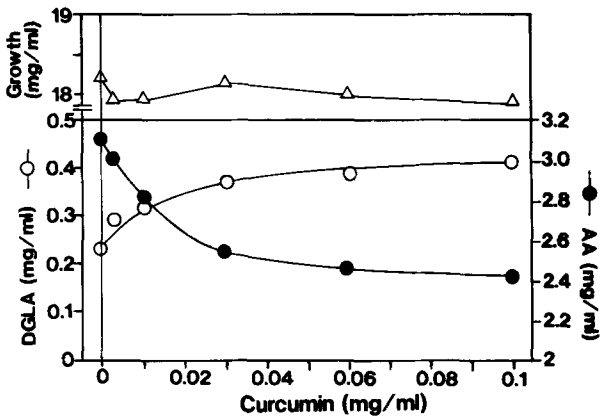

FIG. 2. Effects of curcumin on the production of DGLA and AA by *M. alpina* 1S-4. Curcumin was added as an ethanol solution to sterile medium B at the final concentrations indicated, then *M. alpina* 1S-4 was cultured at 28°C for one week. All value are means of three determinations, the standard deviation being less than 10%.

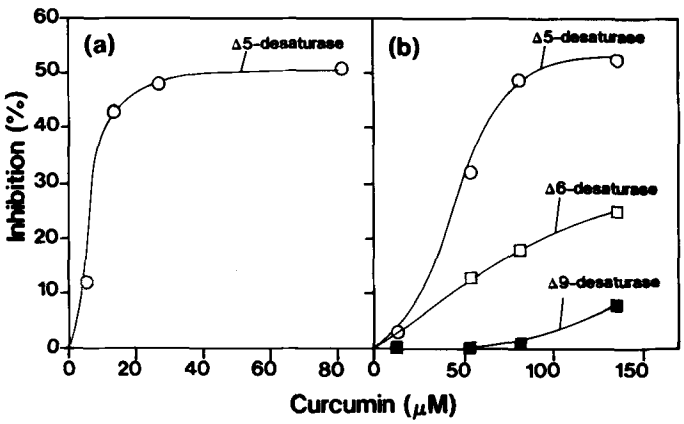

FIG. 3. Effects of curcumin on the Δ5 desaturase activities in cell-free extract of *M. alpina* 1S-4 (a) and rat liver microsomes (b). The reaction was done at 28°C (a) or 37°C (b), using the respective labeled fatty acids as substrate under the conditions described previously (2). The percentage of inhibition was calculated from the amount of radiolabeled fatty acids formed as compared with the control (no curcumin addition). The specific activity of Δ5 desaturase in the fungal cell-free extract was 24 pmol/min/mg protein, and those of rat microsomal Δ5, Δ6 and Δ9 desaturases were 131, 19 and 38 pmol/min/mg protein, respectively. All values are means of three determinations, the standard deviation being less than 10%.

DISCUSSION

These studies were undertaken to screen for Δ5 desaturation inhibitors from natural sources. Since the fatty acid composition can be expected to reflect desaturation activity in cells, we performed the screening by growing a potent AA producer, *M. alpina* 1S-4, in the presence of the test materials, followed by analysis of the fungal fatty acids. We found that curcumin, the major component of turmeric (*Curcuma longta*, L.) (8), mainly inhibited Δ5 desaturation in *M. alpina* 1S-4 fungus. However, in rat liver microsomes, curcumin markedly inhibited Δ6 desaturation. Curcumin has been studied intensively by many investigators and has been shown to have inflammatory activity (9–17), as well as showing inhibitory effects on mammalian 5-lipoxygenase and cyclooxygenase (18). However, as far as we know, there has been no report on its effect on the fatty acid desaturation system.

Except for our earlier paper (2), there has been no report demonstrating the occurrence of desaturase inhibitors in nature. Some unnatural desaturase inhibitors have been synthesized, and most of them are analogs of fatty acids which nonspecifically inhibit any of the desaturases involved in PUFA biosynthesis. For example, several *trans*-octadecenoic acids are competitive inhibitors of Δ9, Δ6 and Δ5 desaturases of rat liver microsomes (19,20). A kinetic study of the inhibition mode of curcumin was not done, but the inhibition is probably not competitive. On a structural basis, curcumin should not compete with any fatty acid for any desaturase.

The enzyme Δ5 desaturase converts DGLA to AA with the result that a precursor for the 1-series prostaglandins is converted to a precursor for the 2-series. Since curcumin inhibited the Δ5 desaturase, it may be able to influence the balance of the two fatty acids and, therefore, alter various metabolic pathways. It would be of interest to investigate the effect of curcumin on the fatty acid desaturation (or biosynthesis) in humans, since curcumin is a component of an edible spice that is widely consumed.

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Nonesterified Fatty Acids in Normal and Diabetic Rat Sciatic Nerve

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Alloxan-induced diabetes in rats results in elevated levels of nonesterified fatty acids (NEFA) in whole sciatic nerve and its endoneurium. Increases in NEFA levels are more pronounced in whole diabetic nerve (40% over control) than in its endoneurial portion (20–30%). Alterations in the composition of phospholipid fatty acids are observed as well, including an increase in linoleate (18:2n-6) in endoneurial phosphatidylethanolamine and a decrease in arachidonate (20:4n-6) in both phosphatidylethanolamine and phosphatidylinositol of diabetic nerve.
Lipids 27, 513–517 (1992).

Peripheral neuropathy is a common complication of human diabetes mellitus as well as of chemically-induced or genetically determined diabetes in experimental animals (1–3). Common functional changes induced by diabetes in peripheral nerve include reduced conduction velocity, action potential and axonal transport, whereas morphological changes can include segmental demyelination (2). The underlying physiological and biochemical alterations induced by diabetes appear to be related to the microenvironment of the nerve, including blood flow and oxygen delivery (4) and changes in *myo*-inositol and inositol phospholipid metabolism (5). Reduced blood flow and hypoxia observed in diabetic rat sciatic nerve (6) were recently correlated with the generation of oxygen free radicals and the accumulation of lipid peroxidation products (7).

Ischemia and hypoxia are often associated with the liberation of fatty acids from complex lipids by the action of lipolytic enzymes, a process which is especially pronounced in the central nervous system (8). In addition, alterations in fatty acid metabolism are an important component of diabetes mellitus (9) and we have previously observed significantly elevated levels of nonesterified fatty acids (NEFA) in the myocardium of alloxan-diabetic rats (10). There exists, however, very little information on the amount or composition of NEFA in peripheral nerve in general (11–13) and none regarding diabetic animals. In order to examine whether changes in nonesterified fatty acids may play a role in the development of diabetic neuropathy, we have measured their levels and composition in the endoneurium and in whole sciatic nerve from normal and alloxan diabetic rats. The results are reported here.

EXPERIMENTAL PROCEDURES

Experimental animals and induction of diabetes mellitus. Male Sprague-Dawley rats of 150–180 g were obtained from a commercial colony (Bio Lab Corporation, St. Paul, MN) and housed in separate cages within a modern animal care facility. All animals had free access to food (Purina #5001, St. Louis, MO) and water throughout the study and were exposed to a 12 h light/12 h dark cycle. Diabetes was induced in each animal by a single injection of 4% alloxan monohydrate (Sigma Chemical Company, St. Louis, MO) in sterile saline into a tail vein at a dosage of 55 mg/kg body weight as described previously (10,14). Control animals received the appropriate volume of sterile saline. Nonfasting plasma glucose levels were measured three days later and at monthly intervals by enzymatic assay (Sigma) coupling the reactions of glucose oxidase and peroxidase. Glycosylated hemoglobin was measured according to the method of Abraham *et al.* (15) using Isolab affinity columns (Isolab, Inc., Akron, OH). Alloxan-injected rats with plasma glucose levels greater than 300 mg/dL and showing little or no weight gain were considered insulin deficient, and saline-injected animals with glucose levels below 150 mg/dL and normal rates of weight gain were used as controls. Any animal not meeting those criteria (*i.e.*, with plasma glucose level between 150 and 300 mg/dL or hyperglycemic but weight gaining) was excluded from the study. These limited criteria for inclusion of animals, used routinely in our studies of experimental diabetes, were selected (10,14) to minimize experimental variations due to differing severities of diabetes which we have shown to affect myocardial complications (16).

In accord with standards of proper animal care, efforts were made throughout the study to minimize or eliminate any unnecessary discomfort to the experimental animals. A diabetic rat of this model will normally remain active and able to groom itself. Food and water consumption and urine output will be above normal. In these experiments, any animal which showed a sudden or significant chronic weight loss, developed anuria, or became lethargic or unable to eat or drink was removed from the study and sacrificed by CO_2 inhalation.

At the intervals described below, diabetic and nondiabetic rats were moderately anesthetized by subcutaneous injection of sodium pentobarbital (50 mg/kg body weight) and brought to full anesthesia by intraperitoneal injection at the same dosage. The skin and superficial fascia of the right thigh of each rat were incised to expose the sciatic nerve from the point where it emerged from the gluteal muscles, past its separation into tibial, peroneal, and sural divisions, to the points where these passed deep to peripheral musculature. This portion was quickly excised and prepared for lipid analysis as described below. The animal was then killed by decapitation while still under anesthesia.

Preparation of sciatic nerve tissue. Experiment 1: Age-matched diabetic and nondiabetic control animals were killed 8 wk after the induction of diabetes. The excised sciatic nerve was washed in saline on cooled (15°C) dental wax, freed of adherent blood vessels and connective tissue, frozen in liquid N_2, weighed, and prepared for lipid analysis. Experiment 2: Age-matched diabetic and nondiabetic control animals were killed 21 wk after induction of diabetes. Upon removal, the nerve was washed in cooled saline, and the epineurium and perineurium were

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Abbreviations: GC, gas chromatography; NEFA, nonesterified fatty acids; TLC, thin-layer chromatography.

stripped away by the procedure of Dyck *et al.* (17). The remaining endoneurium was weighed and prepared for lipid analysis.

Analysis of sciatic nerve lipids. Total lipids were extracted from sciatic nerve endoneurium or total sciatic nerve according to the procedure of Folch *et al.* (18). Briefly, nerve tissue was homogenized in cold methanol containing butylated hydroxytoluene using a Dounce homogenizer and then extracted with chloroform/methanol (2:1, vol/vol) containing 10 μg of heptadecanoic acid as internal standard. The phases were separated by adding 0.2 vol of 0.9% NaCl to the mixture which was again vortexed to remove water soluble contaminants. The residual tissue and upper aqueous layer were re-extracted with chloroform/methanol/water (86:14:1, vol/vol/vol). The pooled chloroform layer was evaporated under N_2 and the lipids redissolved in chloroform for further analysis. Aliquots were assayed for lipid phosphorus according to Bartlett (19).

A portion of the lipid extract was analyzed for NEFA after addition of diazomethane (20) using heptadecanoic acid as an internal standard. Briefly, the lipid extract was dried down under N_2, dissolved in freshly prepared diethyl ether/methanol (9:1, vol/vol), and treated with a solution of diazomethane in diethyl ether for 10–15 min. Fatty acid methyl esters were subsequently purified by silicic acid column chromatography and analyzed by gas chromatography using a Packard (Downers Grove, IL) model 428 gas chromatograph equipped with a Supelco (Bellefonte, PA) 2330 packed column and flame ionization detector. Column temperature was programmed from 180°C to 235°C at 2°C/min and the detector and injection port temperatures were 260°C and 250°C, respectively. The peaks were identified by comparison with standards obtained from NuChek Prep (Elysian, MN) and aliquots were hydrogenated to confirm the identity of unsaturated fatty acids. Peak areas were quantitated with a Spectra-Physics (San Jose, CA) SP4270 computing integrator.

Another portion of the lipid extract was applied to a small column of silicic acid and eluted with 10 mL chloroform (neutral lipids), 20 mL acetone (glycolipids) and 10 mL methanol (phospholipids). The phospholipid classes were quantified by phosphorus assay (19) after separation by two-dimensional thin-layer chromatography (TLC) (21) as described previously (22). For the analysis of their constituent fatty acids, phospholipid classes were isolated by one-dimensional TLC on layers of silica gel H containing 7.5% magnesium acetate using chloroform/methanol/ammonium hydroxide/water (65:35:5:1, by vol) as developing solvent. Choline and ethanolamine phospholipids were further purified by TLC on silica gel H using chloroform/methanol/water (65:25:4, vol/vol/vol). Ethanolamine plasmalogens were hydrolyzed in HCl-saturated diethyl ether, and the resulting lysophosphatidylethanolamines were separated from the nonhydrolyzed diacyl analogs by TLC. Purified phospholipids were transesterified with 0.2 N NaOH in methanol at 45°C for 1 h. Methyl esters were extracted into hexane and analyzed by gas chromatography (GC) as described above.

Statistics. All values between diabetic and non-diabetic groups in Experiments 1 and 2 were compared by two-tailed, unpaired Student's *t*-test, with significance indicated by $P \leq 0.05$.

RESULTS

Effects of alloxan-induced diabetes. As demonstrated by the data of Table 1, diabetic animals exhibited severe hyperglycemia, elevated levels of glycosylated hemoglobin and plasma lipids, increased water intake and essentially no weight gain compared to nondiabetic control rats. The wet weight of the endoneurium was reduced in diabetic animals, but increased moderately when corrected for body weight due to the marked loss of adipose tissue (14,16) which offsets continued growth of lean body mass to yield little or no net weight gain in this model.

NEFA content and composition of sciatic nerve. As shown in Table 2, Experiment 1 demonstrated that the total amount of sciatic nerve NEFA was significantly higher in diabetic rats than in age-matched non-diabetic control animals when expressed relative to either wet

TABLE 1

Effects of Alloxan-Induced Diabetes[a]

	Nondiabetic control	Diabetic
Body weight (g)	625 ± 29	240 ± 63[b]
Weight gain (g/wk)	20.6 ± 1.1	1.7 ± 3.1[b]
Sciatic endoneurium, wet wt (mg)	30.2 ± 4.6	19.8 ± 4.7[b]
Sciatic endoneurium, wet wt/body wt (mg/g)	0.049 ± 0.008	0.085 ± 0.019[b]
Water intake (mL/day)	49.8 ± 11.1	280.9 ± 68.8[b]
Plasma lipid P (μmol/mL)	0.97 ± 0.10	1.67 ± 0.34[b]
Glycosylated hemoglobin (%)	4.98 ± 0.64	16.01 ± 2.60[b]
Blood glucose (mg/dL)	108.3 ± 7.1	533.3 ± 65.0[b]

[a]Measured 21 wk after administration of alloxan (55 mg/kg body wt, i.v.) or saline to 6 wk old rats. Each value represents $\bar{x} \pm$ SD of 4 control and 7 diabetic animals.

[b]Significant difference between control and diabetic group ($P \leq 0.05$).

TABLE 2

Content and Composition of Nonesterified Fatty Acids (NEFA) of Whole Sciatic Nerve from Nondiabetic and Diabetic Rats[a]

	Nondiabetic normal	Diabetic
nmol NEFA per μmol lipid P	14.8 ± 1.9	21.1 ± 1.6[b]
μmol NEFA per g wet tissue	1.22 ± 0.13	2.23 ± 0.31[b]
	mol % FA	
16:0	33.3 ± 0.8	32.4 ± 1.5
16:1	3.0 ± 0.4	2.1 ± 0.02[b]
18:0	14.2 ± 1.3	15.0 ± 1.2
18:1	37.6 ± 0.3	38.5 ± 2.1
18:2n-6	2.1 ± 0.1	2.8 ± 0.5[b]
20:0	1.6 ± 0.1	1.6 ± 0.1
20:4n-6	2.8 ± 0.4	2.4 ± 0.2
22:0	3.1 ± 0.3	3.1 ± 0.2
24:0	2.4 ± 0.3	1.9 ± 0.2

[a]Age, 14.5 wk; duration of diabetes, 8 wk. Each value represents $\bar{x} \pm$ SD of 4 nerve samples.

[b]Significant difference between control and diabetic group ($P \leq 0.05$).

weight or total lipid phosphorus. When the NEFA composition of the nerve was examined, a modest decrease in palmitoleic acid (16:1) and increase in linoleic acid (18:2n-6) were noted in nerve from diabetic animals. The levels of arachidonic acid (20:4n-6) and tetracosanoic acid (24:0) were also reduced although these changes were not statistically significant.

NEFA content and composition of sciatic endoneurium. Based on the results of Experiment 1, Experiment 2 was designed to examine the effects of prolonging the diabetic interval and to focus more specifically on the endoneurial portion of the sciatic nerve by removing the perineurium and epineurium, which are primarily connective tissue. The results are presented in Table 3. Removal of the connective tissue sheaths significantly reduced the total NEFA content in samples from both normal and diabetic animals, although the effect was greater when expressed per μmol of lipid phosphorus, reflecting the relatively large amount of phospholipids in the neurolemma and myelin sheath. Compared to whole nerve, diabetes induced a smaller increase in endoneurial NEFA and considerable variation in their composition.

The phospholipid class composition in diabetic nerve was the same as that of normal nerve with the exception of phosphatidylinositol which was reduced in diabetic nerve (data not shown). This agrees with our previous observations in the streptozotocin diabetic rat model (22). The fatty acid composition of individual endoneurial phospholipids of normal and diabetic nerve is shown in Table 4. Modest differences were observed in fatty acids containing 16 to 20 carbons. Within all of the phospholipid classes examined in this study, of potential significance is the elevation of linoleate (18:2n-6) in phosphatidylethanolamine and the reduction of arachidonate (20:4n-6) in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. As in Tables 2 and 3, the relatively large standard deviations indicated marked within-group

TABLE 3

Content and Composition of Endoneurial Nonesterified Fatty Acids (NEFA) in Nondiabetic and Diabetic Rat Sciatic Nerve[a]

	Nondiabetic normal	Diabetic
nmol/NEFA per μmol lipid P	7.3 ± 1.5	10.1 ± 2.4[b]
μmol NEFA per g wet tissue	0.96 ± 0.17	1.37 ± 0.34[b]
	mol % FA	
16:0	28.0 ± 1.6	31.6 ± 2.5[b]
16:1	5.4 ± 0.4	4.8 ± 0.3[b]
18:0	13.8 ± 0.7	14.6 ± 1.6
18:1	35.6 ± 1.4	33.2 ± 1.3[b]
18:2n-6	2.0 ± 0.4	2.9 ± 0.3[b]
20:0	2.1 ± 0.3	1.9 ± 0.5
20:1	0.5 ± 0.1	0.6 ± 0.1
20:4n-6	3.6 ± 0.8	2.4 ± 0.4[b]
22:0	4.8 ± 0.9	4.1 ± 1.4
24:0	4.0 ± 0.8	4.0 ± 1.2

[a] Age, 27 wk; duration of diabetes, 21 wk. Each value represents $\bar{x}$ ± SD of 7-8 samples prepared from right sciatic nerve.

[b] Significant difference between control and diabetic group ($P \leq 0.05$).

TABLE 4

Fatty Acid Composition (mol%) of Endoneurial Phospholipids from Normal and Diabetic Rat Sciatic Nerve[a]

Fatty acids	Phosphatidylcholine		Phosphatidylethanolamine		Ethanolamine plasmalogen		Phosphatidylinositol		Phosphatidylserine	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
16:0	39.6 ± 2.1	39.3 ± 0.6	13.4 ± 0.3	12.5 ± 1.0	3.8 ± 0.2	5.7 ± 0.4[b]	11.0 ± 0.7	13.3 ± 0.6[b]	1.3 ± 0.07	1.7 ± 0.3
16:1	2.7 ± 0.1	2.0 ± 0.2[b]	2.0 ± 0.1	1.4 ± 0.1[b]	1.4 ± 0.09	1.0 ± 0.1[b]	1.2 ± 0.05	1.2 ± 0.09	0.4 ± 0.01	0.3 ± 0.05
18:0	9.8 ± 0.7	10.3 ± 1.0	12.2 ± 0.6	13.4 ± 1.8	2.2 ± 0.3	4.0 ± 1.4	39.5 ± 2.4	42.5 ± 1.6[b]	18.0 ± 0.6	18.8 ± 0.3
18:1	37.9 ± 0.2	37.2 ± 0.04	55.2 ± 0.3	54.9 ± 1.8	83.0 ± 1.1	78.6 ± 1.3[b]	13.3 ± 0.4	10.26 ± 0.3[b]	53.8 ± 0.8	51.6 ± 0.8[b]
18:2n-6	1.3 ± 0.4	1.8 ± 0.1	1.5 ± 0.1	4.9 ± 1.0[b]	0.7 ± 0.2	0.9 ± 0.01	0.7 ± 0.07	1.1 ± 0.3	trace	trace
20:0	1.2 ± 0.05	1.5 ± 0.2	0.8 ± 0.04	0.8 ± 0.07	trace	trace	0.79 ± 0.04	0.98 ± 0.1	5.0 ± 0.1	5.5 ± 0.05[b]
20:1	0.6 ± 0.03	0.5 ± 0.07	1.3 ± 0.04	1.6 ± 0.2[b]	0.7 ± 0.03	0.6 ± 0.1			1.2 ± 0.07	1.1 ± 0.1
20:4n-6	2.1 ± 0.5	1.1 ± 0.08[b]	5.7 ± 0.2	3.9 ± 0.4[b]	2.8 ± 0.3	3.2 ± 0.1	22.5 ± 1.8	18.0 ± 1.5[b]	2.9 ± 0.1	3.0 ± 0.02
22:0 + 20:3n-6[c]	2.0 ± 0.1	2.6 ± 0.15[b]	1.4 ± 0.06	1.4 ± 0.2	0.4 ± 0.02	0.5 ± 0.1	5.1 ± 0.3	5.1 ± 0.1	9.9 ± 0.6	10.4 ± 0.1[b]
24:0	1.3 ± 0.3	1.6 ± 0.07	1.3 ± 0.5	0.9 ± 0.1	trace	trace	1.4 ± 0.3	1.48 ± 0.19	4.6 ± 0.5	4.6 ± 0.2
22:4n-6	trace	trace	0.8 ± 0.06	0.7 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	0.86 ± 0.12	1.85 ± 0.6[b]	1.8 ± 0.16	1.7 ± 0.14
22:5n-3	trace	trace	0.7 ± 0.03	0.5 ± 0.1	2.0 ± 0.28	2.4 ± 0.3	trace	trace	trace	trace
22:6n-3	0.75 ± 0.1	0.65 ± 0.1	3.1 ± 0.2	2.3 ± 0.6[b]	1.4 ± 0.15	1.9 ± 0.04[b]	2.1 ± 0.4	2.36 ± 0.65	trace	trace

[a] Age, 27 wk; duration of diabetes, 21 wk. Each value represents $\bar{x}$ ± SD of 3-6 samples.

[b] Significant difference between control and diabetic group ($P \leq 0.05$).

[c] Tentative identification.

variations in the effects of insulin deficiency on sciatic nerve lipids.

DISCUSSION

We have shown that alloxan-induced diabetes results in elevated levels of nonesterified fatty acids (NEFA) in rat sciatic nerve. Based on total lipid phosphorous, NEFA levels in both whole nerve and sciatic endoneurium were as much as 40% higher than in control nerves. However, differences between diabetic and control nerve were far less pronounced than previously observed in myocardial tissue whose NEFA levels increased about three-fold within 5 days of alloxan administration (10).

There exists very little information on the amounts and composition of NEFA in the peripheral nervous system. Berry *et al.* (11) reported the NEFA content of cat sciatic nerve as approximately 5 μmol/g wet weight, about twice our present data, and Yao *et al.* (13) found endoneurial NEFA of rat sciatic nerve to be about 43 nmol/mg dry weight. Because sciatic nerve contains 60–70% water (23), our own data would show NEFA levels to be about 2–3 nmol/mg dry weight, at least an order of magnitude lower than those reported by Yao *et al.* (13). NEFA levels are notoriously difficult to determine and are subject to a number of experimental variabilities (10,24). These can include overestimation due to *post mortem* lipolysis or due to transesterification of ester lipids which may be co-purified with NEFA. Methylation with diazomethane avoids this possibility and does not require isolation of NEFA prior to derivatization. We rigorously standardized our procedures of nerve preparation and extraction using low temperatures and minimal time periods between surgery and lipid extraction. It is not clear whether anesthesia or the surgical procedure itself could affect NEFA levels. However, such effects would most likely be the same in normal and diabetic nerve. *Post mortem* lipolysis appears to occur rapidly in the central nervous system (8,23) but was reported to be much slower in peripheral nerve (13). Our present data are in general agreement with the lowest NEFA values obtained for brain and other mammalian tissues (10,25,26). We believe that presentation of NEFA levels on the basis of total lipid phosphorus is more meaningful than data based on wet or dry weight because it takes into account an easily measurable parameter of membrane lipids.

In contrast to diabetic heart (10), the elevation of NEFA levels in diabetic nerve over normal controls was relatively minor. In the endoneurial portion, which is of primary interest as the functional unit of the nerve, this elevation amounted to about 20–30%, was well reproducible and could be reduced to normal levels by insulin treatment (data not shown). However, we also observed some variation of NEFA levels in sciatic nerves of normal rats including a consistent increase with age (data not shown). It is interesting to note that a much greater portion of labeled acetate was found to be incorporated into NEFA rather than more complex lipids by endoneurial preparations from older rats than by those of younger rats (27). It is therefore unlikely that NEFA are involved in the etiology of diabetic neuropathy.

We found only small proportions of polyunsaturated fatty acids among endoneurial NEFA. Thus, the expected increase of linoleic (18:2n-6) acid at the expense of arachidonic (20:4n-6) acid observed in a number of diabetic tissues (28–30) is measurable only among the acyl groups of more complex lipids and occurs together with other alterations in phospholipid fatty acid compositions (Table 4). These data are in general agreement with earlier results obtained with streptozotocin-diabetic rat sciatic nerve (31). Some of the changes in phospholipid acyl groups appear to be due to decreased desaturase activity in diabetic tissue (32,33), including the Δ6 desaturase catalyzing the conversion of linoleic (18:2n-6) to γ-linolenic (18:3n-6) acid (28,33). These relatively minor changes may be important, however, because certain dietary polyunsaturated fatty acids (34,35), including γ-linolenic acid (36), were shown to have beneficial effects in both experimental and human diabetic neuropathy (34–37). Thus, the relationship between endoneurial phospholipid acyl composition and the functional consequences of diabetic neuropathy deserves further study.

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Changes in Fatty Acid Composition of Plasma and Oviduct Lipids During Sexual Maturation of Japanese Quail

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The fatty acid (FA) compositions of plasma and oviduct phospholipids (PL) and triglycerides (TG) were studied throughout the natural sexual development of the Japanese quail. In the oviduct, PL concentration increased rapidly during the period of active oviduct cell proliferation and then remained at a constant level during the phase of cellular hypertrophy. Oviduct and plasma TG concentrations were 2- and 10-fold higher, respectively, in fully developed animals than in immature ones. During natural sexual maturation of the quail, the FA compositions of PL and TG were markedly modified both in plasma and in oviduct. These qualitative changes occurred predominantly during the period of intense cellular proliferation of oviduct cells, and also were observed in immature animals injected with physiological doses of estradiol. In oviduct PL, the proportions of 20:4n-6 and 22:4n-6 decreased significantly (from 20 to 10% and 3.5 to 0.7%, respectively) whereas those of 18:2n-6 increased (from 8.5 to 21%). In contrast, the plasma PL proportions of 20:4n-6, 22:4n-6 and 18:2n-6 were decreased significantly and the percentage of 18:1n-9 doubled, suggesting that the oviduct is able to utilize certain plasma FA to a greater extent than others. Changes in plasma and oviduct lipid composition occurring in the quail during sexual development may be attributed to estradiol, which stimulates hepatic Δ9 desaturase and inhibits the oviduct Δ6 desaturase. The changes in FA composition observed in oviduct phospholipids are discussed in relation to eicosanoid production and cellular proliferation.
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In many animals, including mammals and birds, steroid hormones affect the growth and differentiation of specific target organs, such as the uterus or oviduct, and induce changes in lipid metabolism in these organs. In the uterus, estradiol stimulates the synthesis of all lipid classes (1,2), increases the rate of 20:4n-6 incorporation into phospholipids (PL) and neutral lipids (3) and enhances phosphatidylinositol metabolism (4,5). Estradiol also stimulates phospholipase A_2 activity (6,7) and increases the production of oxygenated eicosanoids (8,9). These effects appear a few hours after hormonal treatment and precede the proliferation of target cells. Prostaglandins and leukotrienes may play various physiological roles in the regulation of parturition in mammalian species, including the vascular changes associated with menstruation in primates (10) and the control of luteolysis and uterine contractility (11). These compounds may also be implicated in the proliferation of estradiol target cells as shown in *in vivo* and *in vitro* studies. Essential fatty acid deficiency delays the onset of puberty in the female rat, probably due to a reduced availability of arachidonic acid (12). The nature of dietary fat influences the developmental growth of the mammary gland in immature estradiol-stimulated mice; animals fed a 19% menhaden oil plus 1% corn oil diet have altered mammae development as compared to controls (13). Linoleic acid stimulates the growth of human breast cancer cell lines in culture (14). This effect is inhibited by n-3 polyunsaturated fatty acids (PUFA) and seems to be more dependent upon the production of leukotrienes than prostaglandins (15).

In the present study, changes in the fatty acid (FA) compositions of plasma and oviduct lipids were examined in Japanese quail during natural and estradiol-induced sexual development in relation to the proliferative state of the tissue.

MATERIALS AND METHODS

Chemicals. Glycerol tri-[1-^{14}C]palmitate (52 mCi/mmol), cholesteryl [1-^{14}C]oleate (50 mCi/mmol), [9-10(n)^{3}H]oleic acid (5 Ci/mmol) and 1-stearoyl 2-[1-^{14}C]arachidonyl phosphatidylcholine (50 mCi/mmol) were purchased from Amersham France (Les Ullis, France). Heptadecanoic and pentadecanoic acids, di-17:0 phosphatidylcholine, tri-17:0 triglyceride, 17:0 cholesteryl ester and standard fatty acid methyl esters (FAME) were from Sigma (St. Louis, MO). All solvents used were reagent grade.

Animals. Female Japanese quail (*Coturnix coturnix japonica*; 17–55-days-old) were maintained in groups at a constant temperature (23°C) on a daily cycle of light (14 h) and darkness (10 h). Chicken powder chow (UAR 115, Villemoisson, France) and water were provided *ad libitum*. Under these conditions, the sexual maturation of the quail started after 21 days and reached the laying state at about 45 days. Hormonal treatment was performed in immature quail (17-days-old) only. Estradiol benzoate (0.05 mg/kg and 0.5 mg/kg dissolved in olive oil) was injected intramuscularly in a total volume of 0.1 mL. Controls received the vehicle alone. Treated and control animals were sacrificed 24 h after the last injection. Animals were fasted for 16 h before being killed by decapitation. Heparinized blood was collected and the resulting plasma was stored at −30°C until analysis. The oviduct was excised and the magnum (secretory portion of the oviduct) was dissected, weighed and used immediately or stored in liquid nitrogen until lipid analysis.

Lipid analysis. The magnum (70–250 mg of pooled or individual tissues) was homogenized using a glass-glass tissue homogenizer (Kontes P22, Poly Labo P. Block, Strasbourg, France) in 6 mL of chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) and internal standards (10–30 μg of 17:0 fatty acid, di-17:0 phosphatidylcholine, tri-17:0 triglyceride and 17:0 cholesteryl ester). The homogenate was kept at 18°C for 30 min

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Abbreviations: BHT, butylated hydroxytoluene; DMA, dimethylacetal; EB, estradiol benzoate; FFA, free fatty acids; FAME, fatty acid methyl ester; GLC, gas-liquid chromatography; MUFA, monounsaturated fatty acids; PG, prostaglandins; PL, phospholipids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TG, triglycerides; TLC, thin-layer chromatography.

and then centrifuged. The resulting pellet was homogenized with 6 mL of chloroform/methanol (2:1, vol/vol) and centrifuged. The two supernatants were combined, and the lipid extract was washed with 3 mL of 0.73% NaCl. The chloroform layer was removed, dried under vacuum and the residue redissolved in an appropriate amount of chloroform/methanol (1:1, vol/vol) containing 10–15 nCi of each radioactive standard listed in the chemical section. Total lipid extracts from 0.5 mL plasma were prepared according to the procedure of Folch *et al.* (16); internal standards (10–30 μg of 17:0 fatty acid, di-17:0 phosphatidylcholine, tri-17:0 triglyceride and 17:0 cholesteryl ester) and radioactive standards were added to samples during the extraction. Control assays (without tissue or plasma) were carried out for each series of experiments.

The various lipid classes were separated by thin-layer chromatography (TLC) using silica gel 60 plates (20 × 20 cm, 0.25 mm, Merck, Darmstadt, Germany) and hexane/diethyl ether/acetic acid (80:20:2, vol/vol/vol) as the developing solvent. The radioactive bands were detected using a Berthold LB 511 (Wildbad, Germany) TLC analyzer, the gel corresponding to each lipid class was scraped off and the fatty acids were directly converted to methyl esters by adding 1 mL of 10% boron trifluoride in methanol to each tube and heating at 90°C for 45 min. The FAME were extracted twice with 2 mL of heptane after adding 1 mL water to each tube. The heptane extract was removed, concentrated under N_2, and a known amount (10–40 μg) of pentadecanoic methyl ester was added to each sample as an internal standard. FAME were analyzed with a Girdel 3000 (Suresnes, France) gas-chromatograph, equipped with a Ross injector that was coupled with a CR 5A integrator (Shimadzu, Kyoto, Japan). The capillary column was a CP Wax 52 CB (0.32 mm × 50 m; Chrompack, Middelburg, The Netherlands), and the temperature was programmed from 160°C to 230°C at 1.2°C/min; helium was used as carrier gas (20 cm/s). FAME were identified by comparison with the relative retention time of known standards. The percentage and concentration of each fatty acid were computed using the internal standard (pentadecanoic acid) and correction for recovery determined for each lipid class by means of 17:0 internal standards.

Statistical analysis. Data are expressed as mean ± SEM. Since fatty acids were measured as proportions, comparison of the different groups could not be done directly by standard methods. Data were transformed according to the following formula $y_i = \log (x_i/x_d)$, where x_d is a fixed component and x_i the percentage of the fatty acid in the study (17,18). For convenience, we chose palmitic acid as x_d. Statistical significance ($\alpha = 0.05$) was tested using Mann-Whitney or Kruskal-Wallis nonparametric rank tests. Non-linear regression analysis of data concerning the FA composition of PL and triglycerides (TG) *vs.* magnum weight was performed with an IBM PS/2 computer using the program COSY (19).

RESULTS

As shown in Table 1, total PL and TG concentrations increased significantly, both in plasma and oviduct, during natural sexual development of the quail. In contrast, plasma and tissue concentrations of free fatty acids remained at a constant level. Tissue and plasma PL and TG concentrations were measured in naturally developing birds to determine whether the observed changes occurred at a particular state of the sexual growth or were continuous throughout the period of sexual maturation. They were plotted (Fig. 1) *vs.* the corresponding magnum wet weight, which is the best criterion to determine the degree of sexual maturation of an individual (20,21). In oviduct, PL and TG concentrations increased only during the beginning of sexual maturation (up to about 300–400 mg magnum weight). Further growth of the tissue was then associated with constant levels of PL and TG. In contrast, a small increase in plasma PL and TG concentrations was observed during the beginning of sexual maturation, whereas these parameters increased rapidly thereafter (up to about 1.5 g magnum weight).

Treatment of immature quail for three consecutive days with a physiological dose of estradiol benzoate (EB; 0.05 mg/kg) resulted in oviduct growth (Table 1). In EB-treated animals, plasma and oviduct PL and TG concentrations were similar to those observed in naturally developing animals exhibiting the same magnum weight (Fig. 1). A higher dose of EB (0.5 mg/kg) induced a drastic increase in plasma and tissue TG levels (Table 1, Fig. 1b). In contrast, plasma and oviduct PL levels were similar (Fig. 1a) in EB-treated and in non-treated animals.

TABLE 1

Lipid Content of Plasma and Oviduct of Immature, Laying and Estradiol-Treated Quails[a]

	Magnum wet weight (mg)	Phospholipids		Triglycerides		Unesterified fatty acids	
		Plasma (μM)	Oviduct (μmol/g)	Plasma (μM)	Oviduct (μmol/g)	Plasma (μM)	Oviduct (μmol/g)
Immature[b] (n = 8)	8.3 ± 0.2	3.05 ± 0.13	11.9 ± 0.3	0.85 ± 0.09	1.45 ± 0.08	0.41 ± 0.03	0.22 ± 0.03
Laying[c] (n = 6)	4650 ± 395	9.42 ± 0.2[d]	18.8 ± 0.8[d]	8.78 ± 0.9[d]	2.94 ± 0.4[d]	0.46 ± 0.05	0.25 ± 0.03
EB 0.05[e] (n = 6)	46 ± 6	3.71 ± 0.40	12.3 ± 0.8	0.82 ± 0.12	1.58 ± 0.17	0.37 ± 0.05	0.27 ± 0.05
EB 0.5[f] (n = 6)	292 ± 49	3.52 ± 0.44	15.6 ± 1.2[d]	11.4 ± 1.5[d]	8.6 ± 1.3[d]	0.43 ± 0.06	0.21 ± 0.04

[a]Results are means ± SEM; n, number of animals per group.

[b]21-Day-old immature quail.

[c]45-Day-old animal at the onset of laying.

[d]Values within a column are statistically different ($P < 0.05$) from those of the immature group.

[e]21-Day-old quail treated with 0.05 mg/kg of estradiol benzoate for three consecutive days.

[f]21-Day-old quail treated with 0.5 mg/kg of estradiol benzoate for three consecutive days.

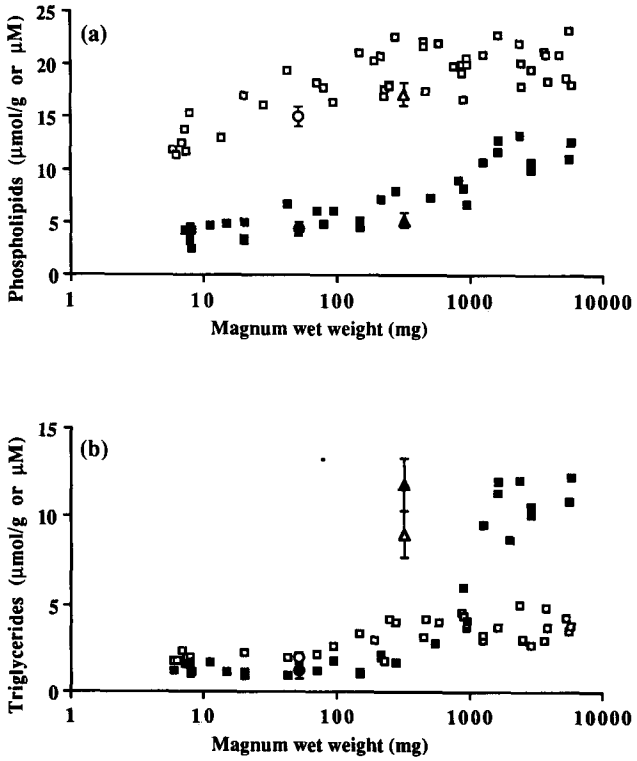

FIG. 1. Total phospholipid and triglyceride concentrations in plasma and oviduct during natural and estradiol-induced sexual development of the quail. (a) Total phospholipid concentration in oviduct (□) and plasma (■). (b) Triglyceride concentration in oviduct (□) and plasma (■). Each point represents an individual animal except for estradiol benzoate-treated birds (○ and ●, 0.05 mg/kg; △ and ▲, 0.5 mg/kg), where the mean ± SEM of six animals are reported. Results are expressed as μmol/g for oviduct and μM for plasma and are plotted against magnum wet weight.

As indicated in Table 2, the FA composition of total PL in both plasma and oviduct changed during the natural sexual development of the quail. In plasma, the most changes occurred in the proportions of monounsaturated fatty acids (MUFA) and PUFA of the n-6 series. The proportions of 16:1n-7 and 18:1n-9 were significantly increased (+211 and +106%, respectively) in laying quail, whereas 16:1n-9 and 18:1n-7 remained at a constant level throughout the maturation period. The proportions of 18:2n-6, 20:4n-6 and 22:4n-6 were significantly reduced (−19, −37 and −55%, respectively). Decreases in the proportions of 18:0 (−19%), 20:5n-3 (−50%) and 22:5n-3 (−48%) also were observed. In the oviduct, the proportion of saturated fatty acids (SFA) was significantly decreased due to a lower level of 18:0 (−36%). Levels of 16:1n-7 and 18:1n-9 were increased (+194% and +14%, respectively) but 16:1n-9 and 18:1n-7 remained unchanged. Total n-6 PUFA was not significantly affected. However, 20:4n-6 (−51%) and 22:4n-6 (−80%) significantly decreased and 18:2n-6 (+146%) increased. The level of 22:6n-3 increased (+41%) while 20:5n-3 and 22:5n-3 decreased, but not significantly. The natural sexual maturation of the oviduct also was associated with an alteration of the dimethylacetal (DMA) composition—18:0 DMA increased (+56%) in laying quail, whereas 16:0 DMA decreased (−48%).

Individual FA composition of plasma and tissue PL also was studied in naturally developing animals from the immature to the laying states (Fig. 2). For convenience, FA showing no trend or those found in very low proportion (≤2%) were not listed. The FA composition of the oviduct PL was closely related to the degree of sexual development of the quail. The percentages of 20:4n-6 and 22:4n-6 decreased continuously as the magnum weight increased (*i.e.*, as the tissue sexually matured); in contrast, the proportion of 18:2n-6 increased throughout the study. The proportion of 18:0 decreased little, the proportion of 18:1n-9 remained virtually constant. For all the FA showing a systematic trend in oviduct PL, various mathematical models were used to describe the results. In all cases, the best model fitting the data was of the form $y = a + be^{kx}$ (where y and x represented the FA percentage and the magnum weight in mg, respectively) and the calculated coefficients of determination were greater than 0.77. According to this model, the rate of change in the proportion of an individual FA occurred mainly during the beginning of the developmental period. DNA synthesis has been shown to exhibit a similar exponential model during the natural maturation of the oviduct (21).

Comparison of plasma (Fig. 2b) and oviduct (Fig. 2a, Table 2) FA profiles showed that some FA (18:0, 20:4n-6, 22:4n-6) varied similarly in plasma and tissue, but 18:2n-6 and 18:1n-9 exhibited a very different pattern. Thus, 18:2n-6 increased in oviduct and decreased in plasma, whereas 18:1n-9 was markedly increased in plasma and was at an essentially constant level in oviduct PL. As indicated in Figure 2 and Table 2, in both plasma and oviduct, estradiol injected in immature quail induced (qualitatively and quantitatively) FA modifications in PL similar to those observed during natural development. Nevertheless, the highest EB dose used (0.5 mg/kg) induced a significant accumulation of 22:6n-3 in oviduct PL (Table 2) which was more pronounced than that observed in naturally developing or laying quail.

The FA compositions of TG in plasma and oviduct during the sexual development of the quail are reported in Table 3 and Figure 3. In the oviduct, the proportions of 18:0 and 20:4n-6 decreased continuously during the natural sexual maturation of the tissue (Fig. 3a) and were significantly reduced (−24 and −74%, respectively) in laying quail as compared to the immature group (Table 3). The proportions of other major FA (principally 18:1n-9 and 18:2n-6) were not significantly modified during this period. The FA composition in plasma TG was quite different than that of the tissue. The proportions of 18:0 and 20:4n-6 decreased in plasma, but to a greater extent (−41 and −90%, respectively) than in the oviduct. Furthermore, 16:1n-7 and 18:1n-9 increased throughout the developmental period (Table 3 and Fig. 3b), and were found in significantly higher proportions in laying quail (+101 and +44%, respectively) than in immature animals. Moreover, the proportion of 18:2n-6 decreased steadily as the maturation of the oviduct progressed and was about half in laying as compared to immature quail. Treatment of immature quail with estradiol, at any dosage used, induced qualitative changes in the FA composition of plasma TG similar to those observed in naturally developing birds presenting the same degree of oviduct maturation (Fig. 3b). In contrast, the FA composition of oviduct TG depended upon the dose injected. A physiological EB dose reproduced the

TABLE 2

Fatty Acid Composition of Total Phospholipids of Plasma and Oviduct of Immature, Laying and Estradiol-Treated Quail[a]

Fatty acid	Plasma				Oviduct			
	Controls (n = 8)[b]	EB 0.05 (n = 6)[c]	EB 0.5 (n = 6)[d]	Laying (n = 6)[e]	Controls (n = 8)[b]	EB 0.05 (n = 6)[c]	EB 0.5 (n = 6)[d]	Laying (n = 6)[e]
16:0	22.5 ± 0.4	24.6 ± 1.0	23.9 ± 1.2	25.4 ± 0.4	22.7 ± 0.3	22.9 ± 0.3	22.9 ± 0.8	26.7 ± 0.6
18:0	21.2 ± 0.6	21.2 ± 0.4	19.4 ± 0.6	17.2 ± 0.6[f]	16.9 ± 0.2	17.2 ± 0.8	16.0 ± 0.3	10.8 ± 0.4[f]
20:0	trace	trace	trace	trace	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
22:0	trace	trace	trace	trace	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.1[f]
SFA	44.0 ± 0.3	46.0 ± 0.6	44.1 ± 0.8	42.6 ± 0.3	40.7 ± 0.5	41.4 ± 0.5	40.0 ± 0.9	38.0 ± 0.7[f]
16:1n-9	trace	trace	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
16:1n-7	0.3 ± 0.1	0.4 ± 0.1	0.8 ± 0.1[f]	1.1 ± 0.1[f]	0.5 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	1.5 ± 0.2[f]
18:1n-7	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	2.1 ± 0.2	2.0 ± 0.1	2.3 ± 0.1	2.2 ± 0.1
18:1n-9	9.0 ± 0.4	11.4 ± 1.0	17.1 ± 1.1[f]	18.6 ± 1.4[f]	15.2 ± 0.2	14.5 ± 0.4	13.7 ± 0.7	17.3 ± 0.7[f]
20:1n-9	trace	trace	trace	trace	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
22:1n-9	trace	trace	trace	trace	0.2 ± 0.1	0.2 ± 0.1	trace	trace
MUFA	12.2 ± 0.5	14.6 ± 1.1	21.4 ± 1.3[f]	24.9 ± 1.3[f]	20.7 ± 0.3	20.5 ± 0.5	19.4 ± 1.0	24.1 ± 0.6[f]
18:2n-6	16.3 ± 1.1	16.1 ± 0.4	15.5 ± 0.1	13.2 ± 0.5[f]	8.6 ± 0.2	11.0 ± 0.5	12.0 ± 0.4[f]	21.1 ± 1.1[f]
18:3n-6	trace	trace	0.2 ± 0.1	0.1 ± 0.1	trace	trace	trace	trace
20:2n-6	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
20:3n-6	0.9 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	0.5 ± 0.1[f]	1.2 ± 0.1	1.5 ± 0.1	1.2 ± 0.6	1.3 ± 0.1
20:4n-6	18.4 ± 0.7	14.9 ± 0.8[f]	11.5 ± 1.1[f]	11.6 ± 0.5[f]	19.9 ± 0.3	17.2 ± 0.4[f]	17.3 ± 1.6[f]	9.7 ± 0.7[f]
22:4n-6	0.6 ± 0.1	0.5 ± 0.1	0.2 ± 0.1[f]	0.3 ± 0.1[f]	3.5 ± 0.1	2.5 ± 0.1[f]	1.9 ± 0.2[f]	0.7 ± 0.1[f]
22:5n-6	0.7 ± 0.1	0.6 ± 0.1	0.4 ± 0.1[f]	0.6 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
n-6 PUFA	37.2 ± 1.0	33.5 ± 1.1	29.1 ± 0.8[f]	26.7 ± 1.1[f]	34.2 ± 0.4	33.7 ± 0.4	33.4 ± 1.1	33.7 ± 1.2
18:3n-3	trace	trace	trace	trace	trace	trace	trace	trace
20:5n-3	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1[f]	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
22:5n-3	1.2 ± 0.1	0.8 ± 0.1	0.6 ± 0.1[f]	0.6 ± 0.1[f]	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	0.9 ± 0.1
22:6n-3	4.3 ± 0.2	4.8 ± 0.3	4.0 ± 0.3	4.2 ± 0.5	2.4 ± 0.4	2.5 ± 0.1	5.8 ± 1.8[f]	3.5 ± 0.2[f]
n-3 PUFA	6.2 ± 0.3	5.8 ± 0.3	5.1 ± 0.3[f]	5.0 ± 0.5	4.1 ± 0.5	4.2 ± 0.2	7.1 ± 2.1[f]	4.6 ± 0.3
20:3n-9	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
16 DMA	trace	trace	trace	trace	1.9 ± 0.2	1.7 ± 0.3	1.4 ± 0.3	1.0 ± 0.1[f]
18 DMA	trace	trace	trace	trace	3.2 ± 0.2	3.0 ± 0.4	3.6 ± 0.4	4.9 ± 0.5[f]

[a] Results are expressed as weight percent, means ± SEM; n, number of animals per group.

[b] 21-Day-old immature quail.

[c] 21-Day-old quail treated with 0.05 mg/kg of estradiol benzoate for three consecutive days.

[d] 21 Day-old quail treated with 0.5 mg/kg of estradiol benzoate for three consecutive days.

[e] 45-Day-old laying quail.

[f] Significantly different from the control group ($P < 0.05$).

changes observed during natural oviduct development, whereas a higher dose induced a significant accumulation of MUFA and a significant decrease of n-6 PUFA.

DISCUSSION

The natural sexual maturation of the female quail is characterized by significant proliferation and differentiation of oviduct cells resulting in a marked increase in oviduct weight (from 15 mg for 21-day-old immature quails to more than 10 g for 45-day-old mature animals). Oviduct growth and maturation are clearly related to increasing levels of circulating estradiol and progesterone (20,21). During this overall period, three phases may be considered and characterized by magnum weight. The first phase (up to about 250–300 mg magnum weight) consists of a very active proliferation of epithelial cells associated with a regular increase in the circulating level of estradiol and a constant low level of progesterone. During the second phase (up to 1.5 g magnum weight), the rate of cellular proliferation decreases while cellular differentiation and egg white synthesis take place. This phase is related to increasing levels of estradiol and progesterone. At the end of this period, cellular proliferation and differentiation are arrested and further growth of the magnum (up to 5–6 g) involves only accumulation of secretory products. This third phase is associated with a constant level of estradiol and increasing values of plasma progesterone.

PL and TG concentrations increased significantly, both in plasma and oviduct, during natural sexual development of the quail (Table 1 and Fig. 1). Our observations are in agreement with studies on mammalian (22,23) and avian (24,25) species showing that sexual hormones influence general lipid metabolism by acting upon both the liver (25–27) and adipose tissue (28) to produce an increase in plasma lipids (22,24). Plasma PL and TG levels increased significantly when plasma estradiol level increased (first and second phase of natural sexual maturation) and were constant thereafter, suggesting that progesterone did not significantly affect these parameters in the quail. Changes

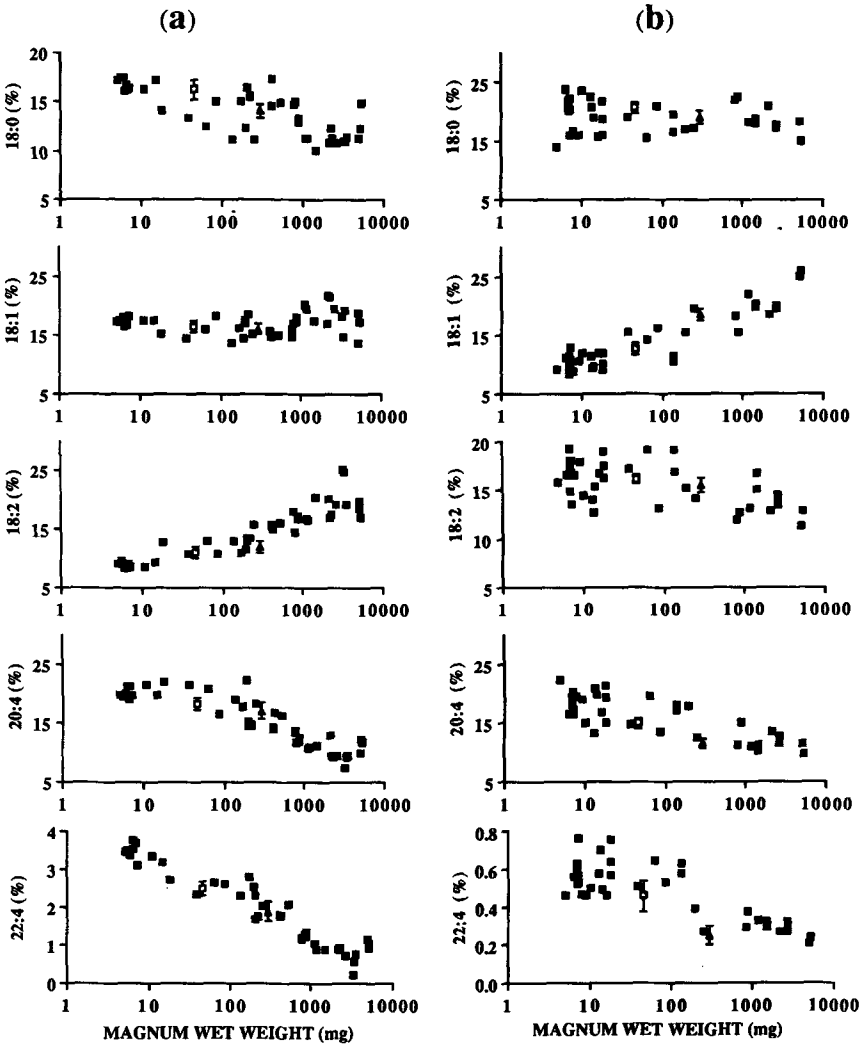

FIG. 2. Fatty acid compositions of total phospholipids of plasma and oviduct during natural and estradiol-induced sexual development of the quail. The percentage of some fatty acids found in the oviduct (a) and plasma (b) are plotted *vs.* the magnum weight during the natural (■) or estradiol-induced (□, △) sexual development. Each point represents an individual animal except for estradiol benzoate-treated birds (□, 0.05 mg/kg; △, 0.5 mg/kg), where the mean ± SEM of six animals are reported.

in the lipid content of the oviduct appeared more rapidly than in plasma. This observation suggests that estradiol has a specific effect on lipid metabolism in the target tissue, and that it takes place during the intense proliferation of the oviduct cells.

The FA composition of plasma lipids was markedly affected, with only minor differences between PL and TG classes. These modifications may be attributed mainly to estradiol, since estradiol injected into immature animals induced qualitatively the same changes as those observed in naturally developing animals, in which both progesterone and estradiol levels are increased (20,21). The proportions of 18:1n-9 and 16:1n-7 were increased, whereas the proportion of 18:0 was reduced, confirming earlier studies in chicken showing that estradiol stimulated hepatic Δ9 desaturase activity (25). Conversely, since 16:1n-9 and 18:1n-7 were not altered, this suggests that the corresponding retroconversion and elongation were not enhanced, but even decreased, by estradiol.

In both PL and TG classes, the oviduct FA composition did not exactly reflect that of plasma lipids, suggesting that the oviduct is able to take up some plasma FA (for example, 18:2n-6 and 18:0) to a greater extent than others (18:1n-9) which were accumulated in plasma. All n-6 PUFA were significantly reduced in plasma, whereas in the oviduct the decreased proportions of 20:4n-6 and 22:4n-6 were compensated by an elevation of 18:2n-6. This suggests that, during the natural or estradiol-induced development of the quail oviduct, the utilization of 20:4n-6 and 22:4n-6 may be greatly enhanced and/or that the metabolic conversion of 18:2n-6 into C_{20} PUFA may be inhibited. Since no accumulation of 18:3n-6 and 20:3n-6 occurred in the tissue, it is likely that Δ6 desaturase activity was not stimulated by estradiol. This may explain why 20:3n-9 was

TABLE 3

Fatty Acid Composition of Triglycerides of Plasma and Oviduct of Immature, Laying and Estradiol-Treated Quails[a]

Fatty acid	Plasma				Oviduct			
	Controls (n = 8)[b]	EB 0.05 (n = 6)[c]	EB 0.5 (n = 6)[d]	Laying (n = 6)[e]	Controls (n = 8)[b]	EB 0.05 (n = 6)[c]	EB 0.5 (n = 6)[d]	Laying (n = 6)[e]
16:0	25.5 ± 0.7	26.6 ± 0.5	27.1 ± 0.6	27.6 ± 0.5	28.3 ± 0.8	26.0 ± 0.2	28.9 ± 0.2	30.0 ± 0.2
18:0	7.3 ± 0.4	7.5 ± 0.5	7.0 ± 0.4	4.3 ± 0.4[f]	13.0 ± 0.7	14.5 ± 0.2	10.0 ± 0.3[f]	9.9 ± 0.4[f]
20:0	trace	trace	trace	trace	0.9 ± 0.2	1.5 ± 0.2	1.1 ± 0.1	0.9 ± 0.1
22:0	trace	trace	trace	trace	trace	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.1
SFA	31.1 ± 0.9	34.6 ± 0.2	33.7 ± 0.5	32.1 ± 0.3	42.1 ± 1.2	42.5 ± 0.2	41.2 ± 0.5	40.9 ± 0.3
16:1n-9	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
16:1n-7	1.7 ± 0.3	1.9 ± 0.4	3.7 ± 0.3[f]	3.6 ± 0.1[f]	2.3 ± 0.4	1.5 ± 0.1	3.5 ± 0.3[f]	2.3 ± 0.2
18:1n-7	1.6 ± 0.2	1.8 ± 0.1	2.0 ± 0.3	2.0 ± 0.3	3.2 ± 0.2	2.9 ± 0.1	3.3 ± 0.2	3.1 ± 0.1
18:1n-9	32.2 ± 0.6	38.7 ± 1.3[f]	42.8 ± 0.8[f]	46.8 ± 0.8[f]	28.4 ± 1.1	26.8 ± 0.4	34.8 ± 1.1[f]	28.1 ± 0.6
20:1n-9	trace	trace	trace	trace	0.9 ± 0.1	1.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.2
22:1n-9	trace	trace	trace	trace	trace	trace	0.1 ± 0.1	trace
MUFA	37.4 ± 0.8	45.1 ± 2.1	50.7 ± 1.1[f]	55.4 ± 0.9[f]	37.8 ± 1.5	34.7 ± 0.5	46.4 ± 1.4[f]	39.2 ± 0.9
18:2n-6	21.5 ± 0.7	15.4 ± 1.6[f]	11.0 ± 1.1[f]	10.8 ± 0.9[f]	15.4 ± 0.6	14.0 ± 0.6	9.1 ± 0.4[f]	14.9 ± 0.9
20:3n-6	trace	trace	0.1 ± 0.1	trace	trace	1.0 ± 0.1	0.5 ± 0.1	0.6 ± 0.2
20:4n-6	4.1 ± 0.1	2.8 ± 0.5	0.6 ± 0.1[f]	0.4 ± 0.1[f]	3.9 ± 0.4	3.2 ± 0.3	1.5 ± 0.1[f]	1.2 ± 0.2[f]
22:4n-6	0.1 ± 0.1	0.1 ± 0.1	trace	0.1 ± 0.1	0.4 ± 0.2	1.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
22:5n-6	trace	trace	trace	trace	trace	trace	trace	trace
n-6 PUFA	26.5 ± 0.8	18.6 ± 2.1[f]	11.7 ± 0.5[f]	11.4 ± 0.7[f]	19.9 ± 0.5	20.8 ± 0.4	12.0 ± 0.6[f]	18.4 ± 0.7
18:3n-3	0.4 ± 0.2	0.2 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	trace	trace	trace	trace
20:5n-3	0.2 ± 0.1	trace	trace	trace	trace	trace	trace	trace
22:5n-3	trace	0.2 ± 0.1	trace	trace	trace	trace	0.1 ± 0.1	0.1 ± 0.1
22:6n-3	trace	trace	trace	trace	trace	trace	trace	trace
n-3 PUFA	0.6 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	<0.3	<0.2	<0.2	<0.3	<0.3
20:3n-9	trace	trace	trace	trace	trace	trace	trace	trace

[a] Results are expressed as weight percent, means ± SEM; n, number of animals per group.

[b] 21-Day-old immature quail.

[c] 21-Day-old quail treated with 0.05 mg/kg of estradiol benzoate for three consecutive days.

[d] 21 Day-old quail treated with 0.5 mg/kg of estradiol benzoate for three consecutive days.

[e] 45-Day-old laying quail.

[f] Significantly different from the control group ($P < 0.05$).

not increased, although 18:1n-9 was increased. In contrast, the lower proportion of 18:2n-6 in plasma lipids may be the consequence of either estradiol induction of the hepatic Δ6 desaturase activity or a specific uptake of this FA by the oviduct.

Extensive literature exists dealing with the metabolism of 20:4n-6 in estradiol target cells (3,29,30). In the models used, 20:4n-6 was mainly converted to prostaglandins and leukotrienes (7,8,31), which have been implicated in specific physiological responses (9,10) and in cellular proliferation (32). Since estradiol stimulates phospholipase A_2 activity (6) and increases 20:4n-6 turnover (3) and oxygenated eicosanoid production (7), all these effects may contribute to the important decrease in 20:4n-6 levels in the oviduct lipids.

Changes in the FA composition in the oviduct were more pronounced during the phase of intense cellular proliferation of the oviduct cells than during the phase of cellular hypertrophy taking place during the final maturation of the oviduct. Metabolites from 20:4n-6 may, therefore, be important for cellular proliferation of estradiol target cells. Two lines of evidence support this hypothesis. First, the addition of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to the culture medium of primary explants of rabbit endometrial cells results in an increase in the rate of cellular proliferation (32). Neither PGE_1 nor PGE_2 affects the rate of proliferation when added alone, but either PGE_1 or PGE_2 reverse the stimulation obtained with $PGF_{2\alpha}$. In this system, PGE_1 increases cAMP, whereas $PGF_{2\alpha}$ stimulates the breakdown of phosphatidylinositol 4,5-bisphosphate (33, 34). Previous studies from our laboratory have shown that, in quail oviduct, the regulation of cAMP level is essential for the control of cellular proliferation. Low levels of cAMP are required for cell replication, whereas high levels are associated with an arrest of cellular proliferation (35, 36). Second, the growth and development of the mammary gland in mice are affected by the type of dietary fat. Animals fed a low fat diet or a fish oil diet had altered mammae development (13). This inhibitory effect has been observed only in estradiol-treated mice, the mammae of which were in a state of intense proliferation. Furthermore, *in vitro* growth of estrogen-responsive cells is stimulated by 18:2n-6 (14,15) and inhibited by 22:6n-3 or 20:5n-3. These effects are dependent upon oxygenated eicosanoid

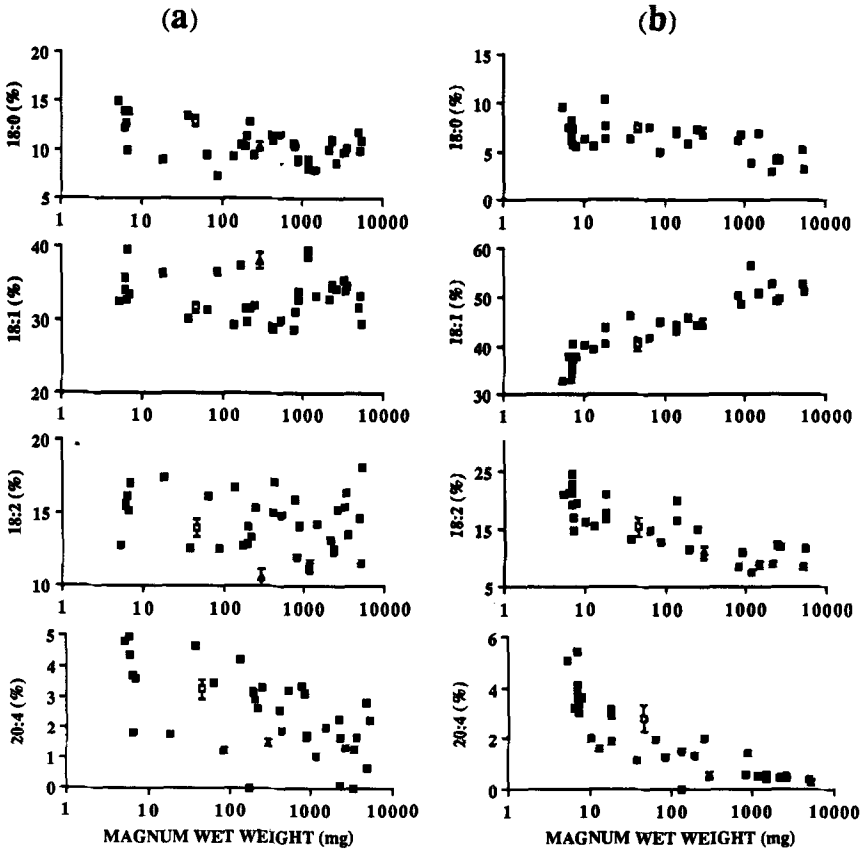

FIG. 3. Fatty acid compositions of triglycerides in plasma and oviduct during natural and estradiol-induced sexual development of the quail. The percentage of some fatty acids found in the oviduct (a) and plasma (b) are plotted *vs.* the magnum weight during the natural (■) or estradiol-induced (□, △) sexual development. Each point represents an individual animal except for estradiol benzoate-treated birds (□, 0.05 mg/kg; △, 0.5 mg/kg), where the mean ± SEM of six animals are reported.

formation since cyclooxygenase and lipoxygenase inhibitors suppress the FA modulation of cell proliferation to varying degrees.

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Dietary Fat and Fatty Acids Modulate Cholesterol Cholelithiasis in the Hamster

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We tested two hypotheses, i) whether the type and the amount of fat in the diet will affect the formation of cholesterol gallstones in the hamsters, and ii) whether palmitic acid, a major fatty acid component of butterfat, can act as a potentiator of cholesterol cholelithiasis in the hamster. Young, male golden Syrian hamsters (Sasco) were fed a semipurified diet containing casein, corn starch, cellulose and cholesterol (0.3%) to which various types and amounts of fat (butterfat, olive oil, menhaden oil, corn oil) were added. All diets contained 2% corn oil to supply essential fatty acids to the growing hamsters. No deaths or illness occurred during the experiment. Animals fed the semipurified diet plus 4% butterfat (group 1) had a gallstone incidence of 63%. Replacement of butterfat with either olive oil, corn oil or menhaden oil prevented the formation of cholesterol gallstones entirely (groups 2–4). When total butterfat was increased from 4% to 8% (group 8), the incidence of cholesterol gallstones increased to 80%. Substitution of 4% olive oil (group 5), corn oil (group 6), or menhaden oil (group 7) for the additional 4% butterfat significantly reduced gallstones to 35%, 45% and 30%, respectively. The replacement of 4% butterfat with 1.2% palmitic acid gave the highest incidence of cholesterol gallstones (95%). These results suggest that butterfat (and one of its components, palmitic acid) intensifies gallstone formation in this model whereas mono- and polyunsaturated fats act as inhibitors of cholesterol cholelithiasis. A fatty acid, possibly palmitic acid, appears to act as lithogen in our model.
Lipids 27, 526–532 (1992).

Cause-and-effect relationships between disease and specific dietary components have been sought over the past two decades. Some types of cancer, particularly colon cancer, have been found to be associated with the ingestion of various types and amounts of fat and/or fiber (1–5). Cholesterol cholelithiasis has been studied in a similar way (6–11) but the dietary factors which influence the course of this disease remain in doubt. Various medical and surgical treatments for cholelithiasis have recently been described, including bile acid therapy (11,12), extracorporeal shockwave lithotripsy (13,14), solvent dissolution (15), and laparoscopic cholecystectomy (16). However, to prevent stone formation, it would be important to clarify the role of the diet in the etiology of cholesterol cholelithiasis.

Many investigators have used hamster models to study gallstone disease (17–23). Because of its size, bile acid composition, and limited hepatic cholesterol synthesis, the hamster is a useful model for human cholelithiasis. Dam (17) fed growing hamsters fat-free diets to obtain cholesterol gallstones. These animals often became ill, lost weight, developed diarrhea and died during the experiment. The dietary manipulations Dam reported, however, suggested a relationship between various fats, carbohydrates and gallstones. A second hamster model (18) was reported to develop cholesterol gallstones on a chow diet supplemented with cholesterol and estrogen. This model was not reproducible in our laboratory (20).

We discovered that young Sasco hamsters develop cholesterol gallstones in a predictable manner when fed a semipurified diet containing 4% butterfat and 0.3% cholesterol (24). This model has enabled us to demonstrate that Sasco hamsters form cholesterol gallstones faster than animals from either Charles River or Harlan Sprague-Dawley when fed our semipurified diet (24). The role of a specific dietary component, namely butterfat, was thought to be essential for stone formation; however, whether the amount or type of fat or a component of the fat could alter gallstone incidence in our model was unclear.

In the present study, we examined, for the first time, whether substitution of various fats for butterfat, including olive oil, corn oil, or menhaden oil, altered the incidence of cholesterol cholelithiasis in the hamster. We also examined if substitution of palmitic acid for butterfat would affect cholelithiasis. All diets contained 2% corn oil to prevent a deficiency of essential fatty acids. Our model previously produced a cholesterol gallstone incidence of 55–65% after a 5- to 10-wk feeding period (24). We also attempted to increase the incidence of gallstones by doubling the amount of butterfat from 4% to 8%. The aim of this study was three-fold: to elucidate the role of i) different fats, ii) total fat, and iii) a specific component of butterfat (palmitic acid), on cholesterol cholelithiasis in Sasco hamsters.

MATERIALS AND METHODS

Compounds. Cholesterol was obtained from Sigma Chemical Company (St. Louis, MO) and found to be greater than 98% pure by gas-liquid chromatography (GLC) after preparation of the trimethylsilyl (TMS) ether derivative. Butterfat, olive oil, corn oil and menhaden oil were obtained from Dyets Premier (Bethlehem, PA). 3α,7α-Dihydroxy-12-keto-5β-cholanoic acid was purchased from Steraloids (Wilton, NH). Palmitic acid (99%) was obtained from Sigma Chemical Company.

Animals and diets. Male golden Syrian hamsters (*Mesocricetus auratus*), weighing between 48 and 50 g, were purchased from Sasco Laboratories (Omaha, NE). All 200 animals were quarantined for one week, during which time they were fed Purina chow and water *ad libitum*. The animals were divided into eight experimental groups and fed the following diets (prepared in pelleted form and color coded by Dyets): group 1 (40 animals) received the standard lithogenic diet containing (g/kg) casein, 200; corn oil, 20; butterfat, 40; corn starch, 434; dyetrose (a soluble

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Abbreviations: CH, cholesterol; CSI, cholesterol saturation index; FT-IR, Fourier transform infrared spectroscopy; GLC, gas-liquid chromatography; LDL, low-density lipoprotein; SPD, semipurified diet; TMS, trimethylsilyl.

starch), 146; fiber, 100; cholesterol, 3; salt mix, 50; vitamin mix, 5; choline chloride, 2. The animals in groups 2, 3 and 4 (20 animals/group) were fed a similar diet with the exception that different types of fat were substituted for butterfat (4%): group 2, 4% olive oil; group 3, 4% corn oil; group 4, 4% menhaden oil. Animals in groups 5–8 (20 animals/group) were given 8% fat in the diet (80 g fat/kg food): group 5, 4% olive oil plus 4% butterfat; group 6, 4% corn oil plus 4% butterfat; group 7, 4% menhaden oil plus 4% butterfat; group 8, 8% butterfat. Animals in group 9 were fed a diet where 4% butterfat (in group 1) was replaced by 1.2% palmitic acid. The fatty acid composition of the diets for groups 1–9 is shown in Table 1. All diets contained 2% corn oil to prevent deficiency of essential fatty acids. The lithogenic diet (group 1) contained a total of 1.3% palmitic acid.

The hamsters were fed the experimental diets and water *ad libitum* for a period of 6 wk and were maintained under an alternating 12-h light and dark cycle. All animals were fasted for 24 h prior to sacrifice to ensure an adequate quantity of bile for analyses. The hamsters were anesthetized with 20–30 mg of ketamine hydrochloride (Bristol Labs, Syracuse, NY) and blood was obtained by cardiac puncture for determination of serum cholesterol; the animals were killed by exsanguination. The gallbladders were exposed and examined visually for the presence of cholesterol gallstones. If present, these stones were easily seen through the gallbladder wall. Cholesterol gallstones were analyzed using diffuse reflectance on a Perkin-Elmer Fourier transform infrared (FT-IR) spectrometer 1710 (Perkin-Elmer, Norwalk, CT) attached to a Perkin-Elmer 7500 computer. Bile was removed and quantitated with a Hamilton syringe (Hamilton Co., Reno, NV) (bile volume ranged from 35–50 μL/animal). All bile samples were centrifuged at 3000 × *g* for 15 min to remove suspended cholesterol crystals; the bile was then immediately divided into aliquots for determination of the biliary lipids. The liver was excised and weighed. An aliquot was removed for determination of cholesterol.

Analytical techniques for bile, liver and plasma. A portion of bile was hydrolyzed in 2 N NaOH for determination of biliary cholesterol. 5α-Cholestane (100 μg/sample) was added prior to GLC (3% SE-30 column) to quantify the cholesterol as the trimethylsilyl ether derivative (24). Biliary bile acids were hydrolyzed with 2 N alkali (24) by autoclaving the samples; the bile acids were extracted and quantitated with a SPB-5 capillary column (15 m, 0.25 μ, Supelco, Bellefonte, PA) attached to a Hewlett-Packard (Avondale, PA) 5890A gas chromatograph controlled by a Hewlett-Packard 3365A workstation with Chemstation software (G12022-10045). Biliary phospholipid concentration was determined using the Biochemical Diagnostics phospholipid kit B (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cholesterol saturation index (CSI) was calculated as described earlier (24,25).

Calculations and statistics. All data are presented as the mean ± standard deviation or standard error of the mean. Differences in gallstone incidence were determined using chi square (26). Statistical differences among groups were calculated using one-way analyses of variance to determine the F statistic. Student's unpaired *t*-test was applied to those values where the F statistic was significant ($P < 0.05$) (26).

RESULTS

The hamsters in each experimental group were fed diets containing either i) 4% fat (groups 1–4), ii) 8% fat (groups 5–8), or iii) 1.2% palmitic acid, in place of 4% fat which was added to the standard semipurified diets. All diets contained in addition 2% corn oil to assure that there was no deficiency in essential fatty acids. The fatty acid composition of all diets (groups 1–9) is summarized in Table 1; the data include the fatty acids present in the dietary fat (if any) and corn oil.

Animals were fed the experimental diets for 6 wk. They were divided into groups (group 1, 40 animals; groups 2–9, 20 animals/group) to assure an adequate number for statistical comparisons. The hamsters gained weight throughout the 6-wk feeding period (Table 2). No animals died during the experiment. The hamsters fed the 4% fat plus 2% corn oil (groups 1–4) gained less weight than animals fed 8% fat plus 2% corn oil (groups 5–8). The weight gain was only moderately related to the food intake which was 9–13 g/d. The largest increase in weight occurred in animals fed 8% butterfat (group 8); the small-

TABLE 1

Fatty Acid Composition of Diets (% by weight of total dietary fat)[a]

	Group number								
Fatty acid[b]	1	2	3	4	5	6	7	8	9
<12:0	6.8	—	—	—	4.0	4.0	4.0	8.1	—
12:0	2.8	—	—	—	1.2	1.2	1.2	2.4	—
14:0	7.5	—	—	6.1	4.5	4.5	8.1	8.9	—
16:0	22.0	12.5	11.2	16.6	18.4	17.6	20.8	24.0	44.0
18:0	8.8	2.7	2.0	3.4	6.5	6.1	6.9	10.1	—
18:1	29.2	57.6	28.5	20.3	46.1	28.7	23.8	29.1	20.0
18:2	21.0	26.4	57.1	21.0	16.6	36.0	13.3	13.3	36.0
Other	1.9	0.8	1.2	32.6[c]	2.7	1.9	21.9[c]	4.1	—

[a]Fat added (%): group 1, butterfat, 4%; group 2, olive oil, 4%; group 3, corn oil, 4%; group 4, menhaden oil, 4%; group 5, butterfat, 4%, + olive oil, 4%; group 6, butterfat, 4%, + corn oil, 4%; group 7, butterfat, 4%, + menhaden oil, 4%; group 8, butterfat, 8%; group 9, palmitic acid, 1.2%. All diets (1–9) contained 2% corn oil. Diets 1 and 9 had similar palmitic acid contents.

[b]The major fatty acids are: 12:0, lauric acid; 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid.

[c]Menhaden oil contains the ω3 fatty acids 20:5 and 22:6.

TABLE 2

Data for Animals at Sacrifice[a]

Group number	Diet	Number of animals	Weight gain[a] (g/6-wk)	Food intake[b] (g/day)
1	Semipurified diet (SPD) + 0.3% cholesterol (CH) + 4% butterfat (lithogenic diet)	40	35 ± 3[c]	10 ± 1
2	SPD + 0.3% CH + 4% olive oil	20	38 ± 3[c]	10 + 1
3	SPD + 0.3% CH + 4% corn oil	20	52 ± 5[d]	10 ± 1
4	SPD + 0.3% CH + 4% menhaden oil	20	29 ± 3[e]	9 ± 1
5	Lithogenic diet + 4% olive oil	20	81 ± 12	13 ± 1
6	Lithogenic diet + 4% corn oil	20	87 ± 12	12 ± 1
7	Lithogenic diet + 4% menhaden oil	20	60 ± 9	13 ± 1
8	Lithogenic diet + 4% butterfat	20	94 ± 16	13 ± 1
9	SPD + 0.3% CH + 1.2% palmitic acid	20	44 ± 10[f]	11 ± 1

[a]All hamsters weighed between 48–49 g at the start of the experiment.
[b]Food was provided to the animals every 4 d. The data represent the average food intake per day throughout the 6-wk experiment. Numbers are mean ± standard deviation. Groups 1–4, 4% fat + 2% corn oil; groups 5–8, 8% fat + 2% corn oil; group 9, 2% corn oil + palmitic acid.
[c]Differs significantly from group 3, $P < 0.02$.
[d]Differs significantly from groups 4–8, $P < 0.01$.
[e,f]Differs significantly from groups 5, 6 and 8, $P < 0.01$.

TABLE 3

Incidence of Biliary Cholesterol Gallstones and Cholesterol Crystals at Sacrifice[a]

Group number	Diet	Cholesterol stones		Cholesterol crystals	
		Number	(%)	Number	(%)
1	Semipurified diet (SPD) + 0.3% cholesterol (CH) + 4% butterfat (lithogenic diet)	25/40	(63)	25/40	(63)
2	SPD + 0.3% CH + 4% olive oil	0/20	(0)[b]	0/20	(0)[b]
3	SPD + 0.3% CH + 4% corn oil	0/20	(0)[b]	0/20	(0)[b]
4	SPD + 0.3% CH + 4% menhaden oil	0/20	(0)[b]	0/20	(0)[b]
5	Lithogenic diet + 4% olive oil	7/20	(35)[c,d]	7/20	(35)[c,d]
6	Lithogenic diet + 4% corn oil	9/20	(45)[d]	9/20	(45)[d]
7	Lithogenic diet + 4% menhaden oil	6/20	(30)[c,d]	6/20	(30)[c,d]
8	Lithogenic diet + 4% butterfat	16/20	(80)	16/20	(80)
9	SPD + 0.3% CH + 1.2% palmitic acid	19/20	(95)[e]	19/20	(95)[e]

[a]Stones were determined at sacrifice at wk 6. See experimental section for details.
[b]Differs significantly from group 1, $P < 0.01$ by chi square.
[c]Differs significantly from group 1, $P < 0.04$ by chi square.
[d]Differs significantly from group 8, $P < 0.02$ by chi square.
[e]Differs significantly from groups 1–7, $P < 0.01$ by chi square.

est increase observed in hamsters fed 4% menhaden oil. [The palmitic acid group (group 9) gained weight at about the same rate as the lithogenic controls (group 1).]

The incidence of cholesterol gallstones and biliary cholesterol crystals is summarized in Table 3. Gallbladders were removed and dissected at sacrifice and visually examined for the presence of stones. Animals fed the lithogenic diet, which contained 4% butterfat (group 1), had a stone incidence of 63%. These stones were composed entirely of cholesterol as seen by polarized light microscopy and confirmed by FT-IR spectroscopy. The substitution of either olive oil, corn oil or menhaden oil (groups 2–4, respectively) for butterfat resulted in a complete prevention of both cholesterol gallstones and crystals (gallstone incidence, 0%). When total butterfat was increased from 4% to 8% (group 8), gallstone incidence increased to 80%. The addition of 4% olive oil, corn oil, or menhaden oil to the lithogenic diet (which contained 4% butterfat, group 1) resulted in a significant decrease of gallstone occurrence (from 80%, group 8) to 35%, 45% and 30%, respectively (groups 5–7) (Table 3). When we replaced the 4% butterfat in the lithogenic diet with 1.2% palmitic acid (group 9), gallstones increased significantly (from 63% to 95%, $P < 0.01$). As in groups 1–4, all gallstones in groups 5–9 were composed entirely of cholesterol, as determined by both polarized light microscopy, FT-IR and GLC of a stone sample (after preparation of the TMS ether derivative). The addition of the various dietary fats in groups 5–7 suppressed gallstone incidence from that obtained with butterfat alone (group 8).

Cholesterol levels in liver, serum and bile were determined at the time of sacrifice; the results are summarized in Table 4. All liver cholesterol levels were greatly elevated above those previously obtained with the cholesterol-free semipurified diet alone [5 mg/g (24)]. The highest liver cholesterol was present in group 5 (lithogenic diet plus 4% olive oil, 108.8 mg/g) and the lowest in group 9 (semipurified diet plus 0.3% cholesterol plus 1.2% palmitic acid, 40.3 mg/g). With either 4% fat (groups 1–4), 8% fat (groups 5–8), or palmitic acid (group 9), the ani-

TABLE 4

Cholesterol Levels in Hamsters at Sacrifice[a]

Group number	Diet	Liver (mg/g)	Serum (mg/dL)	Bile (mg/mL)
1	Semipurified diet (SPD) + 0.3% cholesterol (CH) + 4% butterfat (lithogenic diet)	78.4 ± 2.7	323 ± 11	5.87 ± 0.52[b]
2	SPD + 0.3% CH + 4% olive oil	83.7 ± 3.7	280 ± 10	5.21 ± 0.46
3	SPD + 0.3% CH + 4% corn oil	92.1 ± 3.0	279 ± 8	4.65 ± 0.38[c]
4	SPD + 0.3% CH + 4% menhaden oil	71.7 ± 3.1	379 ± 22[d]	5.08 ± 0.38[e]
5	Lithogenic diet + 4% olive oil	108.8 ± 3.4[f]	353 ± 14	5.96 ± 0.56
6	Lithogenic diet + 4% corn oil	101.0 ± 2.8[g]	309 ± 12	7.88 ± 0.43
7	Lithogenic diet + 4% menhaden oil	74.7 ± 2.6	612 ± 40[h]	7.66 ± 0.43
8	Lithogenic diet + 4% butterfat	76.7 ± 2.1	339 ± 8	9.53 ± 0.63
9	SPD + 0.3% CH + 1.2% palmitic acid	40.3 ± 1.9[i]	375 ± 17	3.34 ± 0.22[j]

[a]See experimental section for details of analyses. Numbers are mean ± standard error of the mean.
[b]Differs from group 8, $P < 0.01$ by t-test.
[c]Differs from group 6, $P < 0.01$.
[d]Differs from groups 1-3, $P < 0.02$.
[e]Differs from group 7, $P < 0.01$.
[f]Differs from group 2, $P < 0.02$.
[g]Differs from group 3, $P < 0.03$.
[h]Differs from groups 5, 6 and 8, $P < 0.01$.
[i]Differs from groups 1-8, $P < 0.01$.
[j]Differs from groups 1, and 5-8, $P < 0.01$.

TABLE 5

Effect of Dietary Fat on Biliary Lipids in the Hamster[a]

Group number	Diet	Biliary lipid composition (mol %)			Total lipid (g/dL)	Cholesterol saturation index
		Cholesterol	Phospholipid	Bile acid		
1	Semipurified diet (SPD) + 0.3% cholesterol (CH) + 4% butterfat (lithogenic diet)	8.5 ± 0.9	17.6 ± 0.8	73.9 ± 1.6	10.5 ± 0.9	1.19 ± 0.12
2	SPD + 0.3% CH + 4% olive oil	8.0 ± 0.4	19.8 ± 0.6	72.2 ± 0.8	9.3 ± 0.8	1.22 ± 0.08
3	SPD + 0.3% CH + 4% corn oil	6.7 ± 0.3	20.1 ± 0.4	73.2 ± 0.6	9.8 ± 0.8	1.01 ± 0.04
4	SPD + 0.3% CH + 4% menhaden oil	6.8 ± 0.3	20.3 ± 0.4	72.9 ± 0.5	10.5 ± 0.7	1.00 ± 0.04
5	Lithogenic diet + 4% olive oil	8.8 ± 0.4	20.1 ± 0.6	71.2 ± 0.8	9.7 ± 0.8	1.35 ± 0.09
6	Lithogenic diet + 4% corn oil	9.4 ± 0.6	21.8 ± 0.6	68.8 ± 0.9	12.1 ± 0.7	1.28 ± 0.09
7	Lithogenic diet + 4% menhaden oil	9.5 ± 0.5	23.0 ± 0.5	67.6 ± 0.8	11.4 ± 0.5	1.25 ± 0.06
8	Lithogenic diet + 4% butterfat	11.8 ± 0.7[b]	21.8 ± 0.4	66.4 ± 0.9	11.5 ± 0.6	1.59 ± 0.09[b]
9	SPD + 0.3% CH + 1.2% palmitic acid	10.3 ± 0.7	18.3 ± 0.8	71.4 ± 1.1	5.0 ± 0.7[c]	1.82 ± 0.13[d]

[a]All bile analyses were carried out from aliquots obtained at sacrifice. See experimental section for details. Numbers are mean ± standard error of the mean.
[b]Differs significantly from group 1, $P < 0.01$ by t-test.
[c]Differs significantly from groups 1-8, $P < 0.01$.
[d]Differs significantly from groups 1-7, $P < 0.01$.

mals given menhaden oil had the highest serum cholesterol levels (group 4, 379 mg/dL; group 7, 612 mg/dL). Biliary cholesterol was elevated above that of animals fed the semipurified diet alone (24) and ranged from a low of 3.34 mg/mL (group 9) to a high of 9.53 mg/mL (group 8).

The biliary lipids and cholesterol saturation indices are summarized in Table 5. The mol% cholesterol was highest in the animals given 8% butterfat (group 8) (11.8). This was the highest mol% cholesterol observed for any hamsters fed in this experiment. Animals fed 4% corn oil (group 3) had the lowest mol% cholesterol (6.7). The mol% phospholipid ranged from 17.6 (group 1) to 23.0 (group 7). All groups had cholesterol saturation indices of 1 or above. The highest value was in group 9 where stone incidence was greatest (CSI, 1.82); in this group biliary lipids were only 5.0 g/dL.

Biliary bile acids were analyzed by capillary gas chromatography after preparations of the methyl ester/acetate derivatives and are summarized in Table 6. Cholic acid was the major component in the bile of each group [range (%), 34.0-50.5]. Chenodeoxycholic acid was most prominent in group 1 (33.2%) and lowest in group 4 (23.9%). Other bile acids included deoxycholic, hyodeoxycholic, murideoxycholic, and allocholic acids. The percent of hyodeoxycholic and murideoxycholic acids tended to be higher with 8% dietary fat. The animals in which palmitic acid (group 9) was substituted for butterfat (group 1) had the lowest bile acid concentration (28.7 mg/mL); however, the

TABLE 6

Effect of Diet on Bile Acid Composition at Sacrifice[a]

Group number	Diet	Total bile acid (mg/mL)	Biliary bile acid composition (%)					
			CA	CDCA	DCA	HDCA	MDCA	Other
1	SPD + 0.3% CH + 4% butterfat (lithogenic diet)	56.2 ± 5.0	34.0 ± 0.9	33.2 ± 1.2	9.3 ± 0.7	8.5 ± 0.5	2.9 ± 0.3	12.1 ± 0.3
2	SPD + 0.3% CH + 4% olive oil	50.0 ± 4.0	36.1 ± 1.0	31.8 ± 0.9	10.2 ± 0.7	6.1 ± 0.4	1.7 ± 0.2	13.6 ± 0.2
3	SPD + 0.3% CH + 4% corn oil	53.3 ± 4.6	36.8 ± 0.8	28.9 ± 0.8	11.9 ± 0.8	7.3 ± 0.5	2.2 ± 0.1	12.9 ± 0.1
4	SPD + 0.3% CH + 4% menhaden oil	56.3 ± 3.6	50.5 ± 1.0	23.9 ± 1.1	8.6 ± 0.4	7.3 ± 0.5	1.7 ± 0.1	8.1 ± 0.4
5	Lithogenic diet + 4% olive oil	50.8 ± 4.1	38.9 ± 0.9	26.4 ± 1.1	12.4 ± 0.6	12.5 ± 0.6	3.2 ± 0.2	5.8 ± 0.6
6	Lithogenic diet + 4% corn oil	61.6 ± 3.6	37.7 ± 1.1	27.0 ± 0.9	12.1 ± 0.5	13.3 ± 0.7	3.1 ± 0.3	6.8 ± 0.5
7	Lithogenic diet + 4% menhaden oil	57.7 ± 2.3	46.2 ± 1.5	25.5 ± 1.4	6.8 ± 0.4	11.9 ± 0.5	2.7 ± 0.3	6.9 ± 0.5
8	Lithogenic diet + 4% butterfat	56.8 ± 3.4	42.3 ± 1.3	26.2 ± 1.2	11.9 ± 0.6	9.9 ± 0.8	2.6 ± 0.4	7.1 ± 0.6
9	SPD + 0.3% CH + 1.2% palmitic acid	28.7 ± 4.1[b]	31.6 ± 1.7	34.0 ± 0.6	9.2 ± 0.7	11.4 ± 1.0	3.3 ± 0.2	11.5 ± 0.7

[a]Bile samples were aliquoted at sacrifice. See experimental section for details. Numbers are mean ± standard error of the mean. CA. cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid. Other bile acids include lithocholic acid, allocholic acid, plus unidentified bile acids, each of which is less than 5%.
[b]Differs significantly from groups 1-8, $P < 0.01$.

percent of the various biliary bile acids was similar to group 1.

DISCUSSION

The hamster model of cholesterol cholelithiasis developed in our laboratory has remained reproducible for several years; gallstone incidence occurs in 45-65% of the animals fed the lithogenic diet (24) for 6 wk. All animals remained healthy during the feeding of this nutritionally adequate diet. The composition of the hamster diet was similar in nutritional content to one described by the National Research Council (27). This assured stone development in healthy animals. Other investigators have used the Sasco hamster to produce gallstones (28,29); the stones produced (cholesterol or pigment) seemed to vary with the dietary components.

The objectives of the current investigation were twofold: to determine if i) the type and the amount of dietary fat, and ii) a particular fatty acid (palmitic acid) affect the development of cholelithiasis. A link between fatty acid(s) and cholelithiasis has been sought but never established (17). The types of diets we used varied in composition with respect to the amount of saturated and unsaturated fatty acids (Table 1). A major saturated fatty acid in all of our diets was palmitic acid (16:0); the dietary concentration of palmitic acid was highest in groups 1 and 8 (as well as group 9) and lowest in the corn oil containing diets (groups 3 and 6). Palmitic acid was known to alter cholesterol metabolism in hamsters by enhancing high density lipoprotein cholesterol and low density lipoprotein receptor mesenger-ribonucleic acid (30,31). There have been no known studies on the effect of palmitic acid on cholelithiasis.

The mechanism by which fats or their individual components alter gallstone formation remains open to questions. In the current experiment, the bile of all hamsters was supersaturated (lithogenic index greater than 1.0) yet only cholesterol (and not pigment) gallstones formed by feeding certain fats to the animals (Table 3). Since the only dietary change among the groups was the administered fat, we assume that this modification affected the incidence of cholelithiasis. Several possibilities for this result can be suggested. First, the bile may contain a protein(s) which prevents (or extends the time of) nucleation of cholesterol crystals. Such proteins have been identified in bile of man (32-35). In man, large quantities of bile are available for analysis, while fasted hamsters, at sacrifice, usually have only 35-50 μL of bile per animal in their gallbladder at sacrifice. Different dietary fats may change the type or amount of bile protein. In studies of only 20 animals/group, the total amount of bile available was insufficient for analysis of the biliary lipids and isolation of individual proteins (or classes of proteins). In this experiment, we chose to determine the biliary lipids and the cholesterol saturation index (CSI). Second, the reason for absence of cholesterol crystals and/or gallstones in bile may be by alterations of the types of cholesterol carriers in the bile (vesicles, micelles, liquid crystals). We did not observe liquid crystals (multilamellar structures in bile under polarized light microscopy); however, vesicles of smaller size (monomeric unilamellar structures) could have been present but remained undetected (36). Vesicular structures solubilize more cholesterol than micelles (36-38). These structures could explain why some hamster biles, even though supersaturated, do not form stones or crystals. Quantitative analyses of vesicles in bile also require 1-2 mL of hepatic bile or 1 mL of gallbladder bile (36-38). The small volume of bile obtained in hamsters thus makes it extremely difficult to determine the vesicular content of bile. Furthermore, the accuracy of the current methodology for quantitation of vesicles and micelles

has been questioned (39). An improved analytical method to separate vesicles from micelles has been described but to date has only been useful for model bile (40).

Nutritional changes can affect cholesterol metabolism as well as cholelithiasis. A recent review by Grundy and Denke (41) pointed out that nutritional factors, such as saturated fatty acids, cholesterol and excess caloric intake, tend to affect serum low-density lipoprotein (LDL) levels in man. Similar factors appear to play a role in increasing the relative cholesterol content (lithogenic index) in bile in our hamster model of cholelithiasis. However, a high biliary cholesterol content *per se* may not be the sole determinant of cholelithiasis. Total fat intake can affect animal weight and can conceivably promote gallstone formation (by increasing cholesterol adsorption). We observed that hamsters ingesting 8% fat gained more weight than those consuming 4% fat (Table 2); food intake (g/d) in both sets of animals was similar. Nevertheless, several groups of animals on 8% fat had a significantly lower incidence of cholesterol stones than others (groups 5 and 6 *vs.* group 8). The gain in weight (44 g/6 wk) of the animals in group 9 (1.2% palmitic acid) was similar to that of the lithogenic control group (group 1, 35 g/6 wk). Gallstone incidence was 95% in group 9 and 63% in group 1. Thus, weight gain seemed to be unrelated to the development of cholelithiasis. The gain in total body weight, as well as total caloric intake alone, does not seem to predict the development of stones in this model.

Interestingly, animals in group 9 where total fat intake was only 3.2% (2% corn oil and 1.2% palmitic acid) had the highest incidence of cholesterol gallstones as well as the highest cholesterol saturation index (1.82). The total lipids in the bile of animals in group 9 were only 5.0 g/dL, about one-half of the value obtained for the control group (group 1, 10.5 g/dL). The decreased concentration of bile acids and phospholipid in the presence of elevated biliary cholesterol produced an elevated cholesterol saturation index which may explain the enhanced lithogenic stimulus exerted by palmitic acid, at least in part. This is the first report that a specific fatty acid caused a very high incidence of pure cholesterol gallstones. It has been reported that palmitic acid affects serum proteins, *i.e.*, it enhanced high density lipoprotein production (30). In addition, biliary phospholipids may alter cholesterol nucleation (42). Since different dietary fats are known to alter the fatty acid composition of biliary phospholipid in prairie dogs (43), it is interesting to speculate that a similar phenomenon may be occurring in this hamster model. It is also possible that in our model palmitic acid may affect the concentration or composition of nucleating/antinucleating proteins in bile leading to increased cholelithiasis. Further studies are needed to clarify these points.

Cholesterol in various body tissues increased with the intake of dietary fat. The amount of cholesterol in liver, plasma and bile of Sasco hamsters fed a semipurified diet containing no added dietary fat was 5.0 mg/g, 182 mg/dL and 1.84 mg/mL, respectively (25), well below the values in animals given dietary fat and cholesterol. There was no correlation between the concentration of cholesterol in liver or serum and the incidence of cholesterol gallstones. For example, hamsters in the lithogenic control group (group 1) had a mean liver cholesterol level of 78.4 mg/g and the incidence of cholesterol stones was 63%. Animals in groups 2–4 had no gallstones, yet liver cholesterol ranged from 71.7 to 92.1 mg/g. In general, serum cholesterol increased when dietary fat was increased (groups 1–4 *vs.* groups 5–8). In all groups, biliary cholesterol levels were higher and bile was supersaturated with cholesterol when dietary fat was increased, but only certain fats potentiated the formation of gallstones. We suggest that under the experimental conditions employed the concentrations of cholesterol in liver and serum were not predictive of relative biliary cholesterol concentrations, cholesterol saturation indices, or cholelithiasis.

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Changes in Blood Lipids and Fibrinogen with a Note on Safety in a Long Term Study on the Effects of n-3 Fatty Acids in Subjects Receiving Fish Oil Supplements and Followed for Seven Years

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The present study was designed to assess the effectiveness of the n-3 fatty acids in modifying serum total, low density lipoprotein and high density lipoprotein (HDL) cholesterol, as well as serum triglycerides, over a seven-year period. Changes in plasma fibrinogen were recorded and long term safety assessed. A total of 365 subjects with ischemic heart disease (IHD), hyperlipidemia or a strong family history of IHD had their diet supplemented with MaxEPA (Seven Seas Ltd., Hull, England) fish oil containing 18–19% eicosapentaenoic acid. Venous blood samples were taken at regular intervals for lipid and fibrinogen assays and routine clinical chemistry and hematological profiling. Current medication was recorded and no further dietary modification was attempted. Triglyceride and fibrinogen were significantly reduced, whereas a significant reduction in total cholesterol occurred only in the subjects with a pre-oil level > 6.5 mmol/L. HDL cholesterol significantly increased over the study period. Clinical chemistry and hematological profiles were not adversely affected, and platelet count did not change significantly. The type of lipid changes observed were those usually considered antiatherogenic. Reducing fibrinogen may result in beneficial changes in the pathological processes leading to thrombotic occlusion. The consumption of MaxEPA by our patients over a seven-year period did not indicate any adverse effects.
Lipids 27, 533–538 (1992).

In recent years, much attention has been focused on dietary fish and fish oil as a means of improving various coronary heart disease risk factors (1). A high dietary intake of n-3 fatty acids is thought to be responsible for the low incidence of ischemic heart disease in Eskimos living in the Umanak district of Greenland (2). A low incidence of ischemic heart disease also has been reported in coastal-dwelling Turks and Japanese populations consuming a diet rich in n-3 fatty acids (3–6). Blood lipids, in particular, appear to be altered by the regular consumption of n-3 fatty acids. In 1980 we first reported (7) a significant reduction in serum triglyceride in normal volunteers taking MaxEPA (Seven Seas Ltd., Hull, England) a natural marine triglyceride rich in eicosapentaenoic (EPA) and docosahexaenoic acids (DHA). This finding was subsequently confirmed by both open (8–10) and controlled studies in healthy subjects and in patients with ischemic heart disease (11,12). In a further double-blind crossover study on hypertriglyceridemic patients, many of whom suffered previous myocardial infarcts, we demonstrated a significant superiority of n-3 over n-6 fatty acids in reducing serum triglyceride and total cholesterol and increasing high density lipoprotein cholesterol (HDL-C) (13). The bleeding time in Eskimos has been shown to be higher than in the Danes, a group with which the Eskimos have been compared (14,15). Other work has demonstrated an increased bleeding time after the consumption of fish oil in British subjects with ischemic heart disease (16). Re-occlusion after angioplasty has been significantly reduced in patients taking MaxEPA in addition to aspirin and persantin (17) and, indeed, many of the reported properties of fish oil would suggest a potential role in the prevention of coronary thrombosis. This open prospective study was designed to answer two important questions. What are the long-term effects of regular fish oil supplements to the diet on serum triglyceride, serum total cholesterol, low density lipoprotein (LDL) cholesterol and high density lipoprotein (HDL) cholesterol, as well as plasma fibrinogen and platelet count? Secondly, are there any undesirable long-term side effects as judged by clinical chemistry and hematology profiling?

PATIENTS AND METHODS

Three hundred and sixty-five subjects participated in the study (304 males and 61 females), with an age range of 18–76 years and a median age of 51 years. Of these, 47.2% had suffered a myocardial infarction and 1.9% had had a stroke prior to joining the study. A total of 49.2% had angina pectoris as assessed by history of symptoms, 3.8% diabetes mellitus and 6.1% intermittent claudication. Because most of these patients (73%) had hyperlipidemia and, in view of the pre-existing other high risk factors, it was not considered ethical to give them a placebo; hence the study was conducted as an open trial. Hyperlipidemia was defined as a triglyceride level in excess of 2.0 mmol/L and/or a total cholesterol level in excess of 6.5 mmol/L. The data reported on serum lipids covers a seven-year period. Plasma fibrinogen was measured by an outside laboratory, and at four years the method of measurement was changed. The measurement of this parameter was therefore discontinued. Seventy-five subjects withdrew from the study over seven years. The commonest reasons for withdrawal were moving away from the area and dislike of the taste of the fish oil, which was taken by spoonful since capsules of MaxEPA were not yet available. On joining the study, 199 patients were receiving medication for cardiovascular disorders, including treatment for angina and hypertension. Nine had been taking clofibrate but discontinued this before joining the study. No patients taking aspirin or lipid lowering medication were included in the study. Patients joined the study throughout the seven years. The 365 patients were studied for 1 mo; 343 for 3 mo; 300 for 6 mo; 258 for 12 mo; 216 for 18 mo; 204 for 24 mo; 163 for 30 mo; 146 for 36 mo; 126 for 42

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Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; FA, fatty acid; GK, glycerokinase; HDL, high density lipoprotein; HDL-C, high density lipoprotein cholesterol; IHD, ischemic heart disease; LCAT, lecithin:cholesterol acyltransferase; LDH, lactate dehydrogenase; LDL, low density lipoprotein; LDL-C, low density lipoprotein cholesterol; MaxEPA, refined triglyceride concentrate from marine body oils; NADH, nicotinamide adenine dinucleotide (reduced form); PK, pyruvate kinase; VLDL, very low density lipoprotein.

mo; 109 for 48 mo; 84 for 60 mo; 61 for 72 mo; and 40 for 84 mo. Data from patients dropping out of the study were not included.

MaxEPA. The oil used in this study was a refined natural triglyceride concentrate, deodorized and blended from marine body oils. Dodecyl gallate (100 ppm) was added to the oil to retard oxidation and α-tocopherol acetate (1000 ppm was present to reduce the risk of vitamin E deficiency in the recipient. An advantage of this particular fish oil was the low concentrations of vitamins A and D present. The concentration of vitamin A was less than 100 IU/g, and less than 15 IU/g of vitamin D. The MaxEPA used here contained 18.6% EPA and 12.1% DHA.

Blood sampling. Serum for lipid and routine biochemical estimations was separated from venous blood withdrawn with the minimum of stasis after a 12–14 h fast. Blood for hematological estimations was collected into ethylenediaminetetraacetic acid (EDTA). Two blood samples for lipid measurement were taken at seven-day intervals before supplementation of the diet with MaxEPA, and further samples were taken at 1, 3, 6, 12, 18, 24, 30, 36, 42, 60, 72 and 84 mo after starting MaxEPA. In order to avoid diurnal lipid changes, all blood samples were taken between 9:00 and 10:00 a.m.

Dose. During the first year of study, each participant was asked to take 10 mL of MaxEPA (1.8 g EPA) twice daily with food. No further modification of diet was attempted and, to try to avoid the complicating effects of other, concomitant dietary manipulations, patients were encouraged to stay on the same diet as when they first joined the study. Subsequently, the dose was reduced to 10 mL once daily.

Laboratory assays. The method chosen for the measurement of serum triglyceride in the present study was the enzymatic assay after saponification with ethanol/potassium hydroxide (18–20). The glycerol present in serum before and after hydrolysis with ethanol/potassium hydroxide was converted to glycerol-3-phosphate and adenosine diphosphate (ADP) with adenosine triphosphate (ATP) and glycerokinase (GK) (ATP:glycerol 3-phosphotransferase). The ADP reacts with pyruvate kinase (PK) (ATP:pyruvate 2-*O*-phosphotransferase), and added phosphoenolpyruvate to give pyruvate which is reduced to lactate with reduced nicotinamide adenine dinucleotide (NADH) with lactate dehydrogenase (LDH) (L-lactate:NADH oxidoreductase). Triglyceride was determined by direct measurement of free glycerol which was subtracted from the total glycerol measured after alkaline hydrolysis of serum. The difference corresponds to the neutral fat content. The determination of triglyceride is dependent on a double enzymatic measurement of glycerol. No significant interference was observed from mono- and diglycerides which are present in the serum only in trace amounts. The decrease in extinction due to the oxidation of NADH was measured at 340 nm. Total serum cholesterol was measured by an enzymatic method using cholesterol oxidase (21–23). Esterified cholesterol is broken down into free cholesterol and fatty acid by enzymatic cleavage with cholesterol esterase. Cholestenone and hydrogen peroxide are produced as a result of the action of cholesterol oxidase on cholesterol. The amount of hydrogen peroxide produced is directly proportional to the amount of cholesterol present in the serum. After addition of color reagent the absorbance is read at a wavelength of 500 nm. The reagents used in both the above methods were supplied by Boehringer Mannheim U.K. Ltd. (Lewes, England). HDL-C was measured by the method described above after polyanion precipitation of the apoB-containing lipoproteins (very LDL, LDL) with sodium heparin and manganese chloride (24,25). Failure of the precipitate to sediment occurred in samples containing very high levels of very low density lipoprotein (VLDL). In order to measure HDL cholesterol in these samples it was necessary to dilute the serum with an equal volume of 0.01 M Tris-HCl buffer, pH 7.6, after which the measurement was made as described above. Polyacrylamide gel electrophoresis was used on both precipitate and supernatant to ensure complete and specific isolation of HDL. To prospectively assess the long-term safety of MaxEPA, routine clinical chemistry and hematological profiles were measured by the respective laboratories of this hospital using the Technicon H6000 and Vickers SP120 instruments (Technicon Co., Tarrytown, NY). Platelets were counted on a Coulter counter and plasma fibrinogen was measured by the Coagulation Laboratory (26). The Friedewald formula (27) was used to calculate LDL cholesterol, namely LDL-C = (total cholesterol − HDL-C) − triglycerides ÷ 2.19 mmol/L.

Statistics. A method based on piecewise linear interpolation (28), carried out for each individual patient, was used on each of the following variables—serum triglyceride, total cholesterol, HDL-C, LDL-C and plasma fibrinogen.

RESULTS

Serum triglyceride levels in the group of subjects with raised baseline levels (mean 3.76 mmol/L) fell significantly within one month, as did the triglyceride levels in the group with "normal" baseline levels (mean 1.35 mmol/L). In both groups the changes were significant at the 0.1% level. This highly significant decrease in triglyceride continued to the end of the study at seven years. Normal levels were defined as < 2.0 mmol/L. The results are shown in Figure 1.

Serum cholesterol in the group of subjects with raised baseline levels (mean 8.15 mmol/L) was reduced at 1 mo by 0.44 mmol/L, and by 3 mo had reached 0.54 mmol/L (significant at 0.1% level). At the end of seven years the reduction in cholesterol was 1.25 mmol/L. Normal levels were defined as 3.8 to 6.5 mmol/L. The group with a baseline < 6.5 mmol/L (mean 5.64 mmol/L) showed small increases at months 18 to 36, but this change was not significant at the 5% level. However, by the end of the study the cholesterol level in this group had fallen by 0.4 mmol%L (not significant). The results can be seen in Figure 2.

At each timepoint mean HDL cholesterol levels showed an increase from baseline levels (mean 1.22 mmol/L). These increases are shown in Figure 3. Furthermore, at each timepoint a majority of subjects were found to have increased levels of HDL cholesterol (significant at 0.1% level for each timepoint). The magnitude of change did not appear to differ greatly between the group with low baseline levels and the group with normal baseline levels. At each timepoint, mean LDL cholesterol levels demonstrated little or no change from the baseline levels

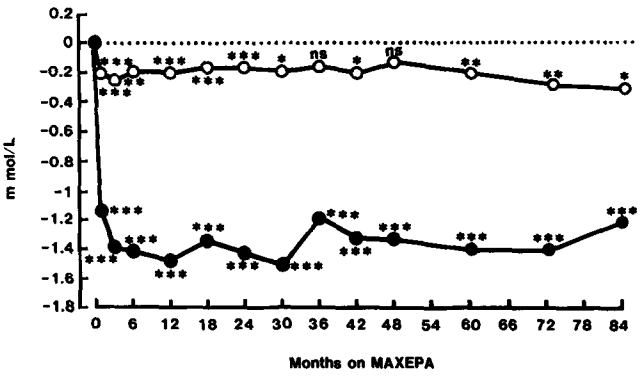

FIG. 1. Serum triglycerides before and during treatment with MaxEPA are shown as a mean change from baseline expressed in mmol/L. The mean level of the subjects with a pre-treatment triglyceride of < 2.0 mmol/L as illustrated by an open circle demonstrates a reduction significant at 0.1% level (***) for the first two years of study, after which changes show significance at a 1% (**) or a 5% level (*). No significant change is apparent at 36 and 48 mo. Changes observed in the subjects with a mean triglyceride level of > 2.0 mmol/L (●) indicate a reduction with significance at the 0.1% level (***) throughout the seven years of study.

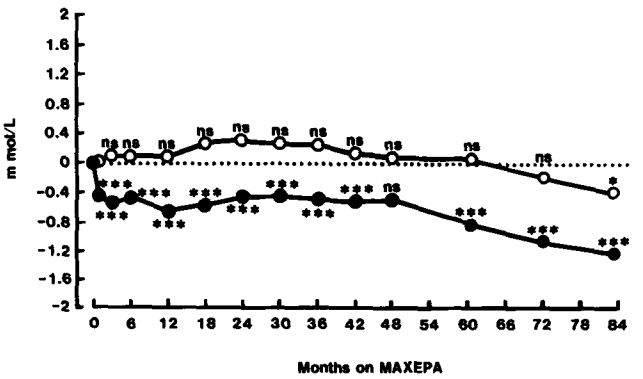

FIG. 2. The mean total serum cholesterol for two groups of subjects before and during seven years of treatment with MaxEPA is shown. In the group with a pre-treatment level of < 6.5 mmol/L (○) no significant change was observed until the final point at 84 mo, when a reduction in cholesterol was observed with a significance at 5% level. However, the group of subjects with a pre-treatment mean total serum cholesterol > 6.5 mmol/L (●) experienced a reduction in cholesterol significant at 0.1% level (***), at all except the 48 mo point during study.

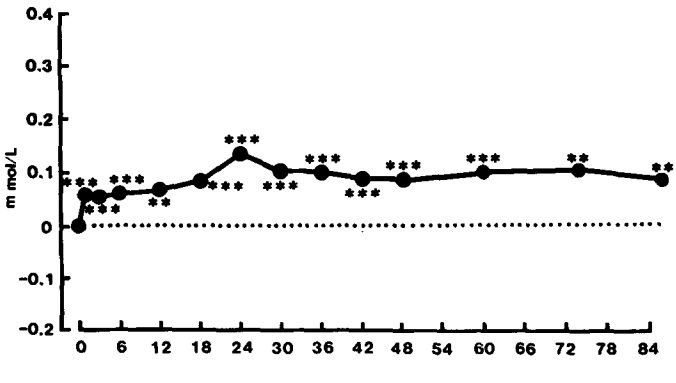

FIG. 3. The high density lipoprotein cholesterol increased within a few weeks of starting treatment with MaxEPA, and at most timepoints the change was significant at 0.1% level (***). At points 12, 72 and 84 mo the increase was significant at a 1% level (**).

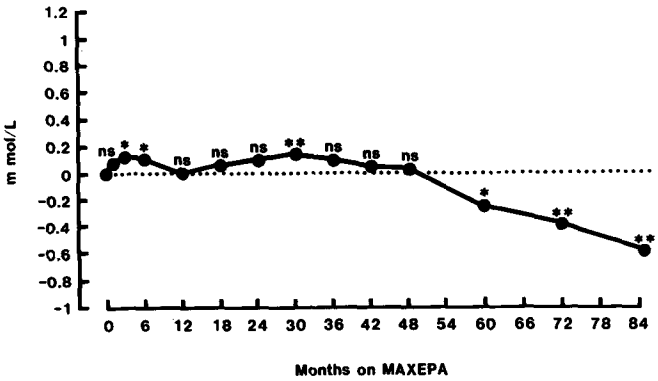

FIG. 4. Low density lipoprotein cholesterol increased at 3, 6 and 30 mo after starting treatment with MaxEPA with significance at 5% (*), 1% (**) levels, respectively. At all other points no significant change was observed until 60 mo through to the end of the study when a significant decrease in LDL cholesterol was apparent. At 60 mo this change was significant at 5% (*) level, at 72 and 84 mo the change was significant at a 1% (**) level.

with the exception of 3, 6 and 30 mo, where a significant increase occurred at the 5% level at the first two points and at 1% at the latter. However, at the end of seven years the LDL cholesterol had fallen by 0.6 mmol/L (significant at 1% level). Changes are shown in Figure 4.

Plasma fibrinogen levels demonstrated mean reductions from baseline levels at each timepoint and these tended to increase in magnitude as time progressed (Fig. 5). After 1 mo, the mean fibrinogen level was reduced by 0.14 g/L, at 6 mo the observed mean reduction from the baseline levels was 0.33 g/L, and at 48 mo the mean reduction was 0.89 g/L (significant at the 0.1% level). No significant change was observed in the mean platelet count during this study (Fig. 6). At the start of the study MaxEPA was not available in capsule form and it was not possible to find a placebo oil with similar taste. It was therefore decided to conduct an open study with a control group. With the exception of taste, as indicated by a number of patients withdrawing from the study, MaxEPA was found to be without apparent side-effects over seven years of medication. Although up to 17.5% of patients had minor disturbances of routine chemical and hematological parameters, none had more than two changes outside the normal range (Table 1). The changes were small and inconsistent and were felt to be of no significance. No change in any parameter could be attributed to MaxEPA.

DISCUSSION

A recent editorial sounded a cautionary note on both the safety and long-term efficacy of fish oil in lowering plasma triglyceride (29). A study using a different fish oil concentrate (Omega-500, Omegacaps, St. Louis, MO) 15 g daily had failed to sustain the initial reduction in plasma triglyceride in 16 patients over 6 mo of study (30). Our much longer study, with larger numbers of patients, found MaxEPA to be safe and to show a persistent reduction in triglyceride concentrations, which was as effective after seven years as during the initial period of study. The only side effect to lead to a number of patients withdraw-

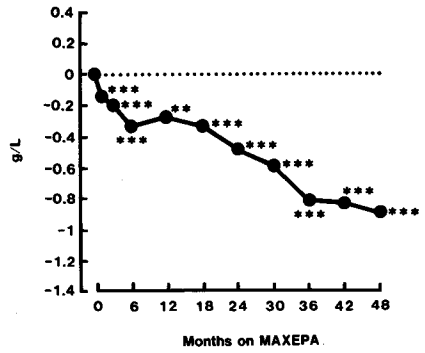

FIG. 5. Plasma fibrinogen, measured for the first 48 mo after starting treatment with MaxEPA, fell significantly at each timepoint. Only at 12 mo was the change significant at the 1% level (**), at every other point the significance was at the 0.1% level (***).

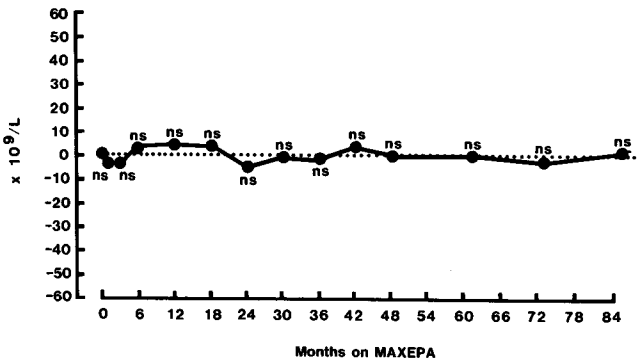

FIG. 6. Platelet numbers were reduced during the first three months after starting treatment with MaxEPA. This reduction did not reach significance. For the remaining period of study there was not significant change in platelet count, which remained close to the baseline level.

ing from the study was the taste of the oil. We cannot explain the reduction in long term efficacy in the Omega-500 trial, where the maintenance dose of n-3 fatty acids was 3.9 g/day. One possible explanation for these differences might lie in the different composition of fish oil produced by different manufacturers. The mechanism by which fish oil reduced plasma triglyceride has been discussed at length elsewhere (16,31–33).

Hypertriglyceridemia is an univariate risk factor for ischemic heart disease, but its role as an independent risk factor is still controversial. In general, hypertriglyceridemia combined with low HDL cholesterol suggests an increased risk of coronary disease. However, a strong relationship between non-fasting VLDL levels and cardiovascular mortality in both men and women has been reported (34). This suggests that triglyceride-rich lipoprotein in random samples is an important predictor of both total and cardiovascular mortality.

Hypertriglyceridemia also has been associated with depressed fibrinolysis, an important factor in ischemic heart disease (35). An association between hypertriglyceridemia and increased plasma concentrations of fibrinogen has been reported to be partially corrected by reducing plasma triglycerides (36). Most myocardial infarcts are the result of thrombotic occlusion. A number of prospective studies have shown plasma fibrinogen to be a highly significant risk factor in the development of coronary heart disease (37–41). In one of these studies (41), platelet aggregability was strongly associated with plasma fibrinogen concentration. A strong correlation has been reported between decreased red cell filterability, high triglyceride, high fibrinogen and low HDL-C levels. The present work suggests a positive correlation between triglyceride and fibrinogen. Reducing triglyceride and maintaining a low plasma level of fibrinogen may result in a lessening of its adverse effects on hemorrheology and damage to the arterial wall. The foregoing would appear to suggest a link, directly or indirectly, between serum triglyceride, plasma fibrinogen and platelet aggregation

TABLE 1

Clinical Chemistry and Hematological Measurements on Patients[a]

Variable (normal range)	No. of patients	No. of patients with at least one value below range	No. of patients with at least one value above range
Sodium (134–144 mmol/L)	211	7 (3.3%)	23 (10.9%)
Potassium (3.8–5.2 mmol/L)	211	15 (7.1%)	35 (16.6%)
Calcium (2.2–2.6 mmol/L)	211	18 (8.5%)	21 (10.0%)
Creatinine (35–120 mmol/L)	211	5 (2.4%)	37 (17.5%)
Urea (2.8–8.0 mmol/L)	211	10 (4.7%)	15 (7.1%)
Albumin (36–50 mmol/L)	209	2 (1.0%)	20 (9.6%)
Bilirubin (1–24 mmol/L)	207	0 (0%)	13 (6.2%)
Leucocytes (3.5–11.5 10^9/L)	283	7 (2.5%)	14 (4.9%)
Hemoglobin (F: 12.3–16.5 g/dL) (M: 13.0–18.0 g/dL)	283	15 (5.3%)	19 (6.7%)

[a]No patient had more than two results outside normal range.

which would not only lead to a hypercoagulable state but might also stimulate atherogenesis. It is suggested that the pathological processes leading to thrombotic occlusion may be modified beneficially by increasing the level of n-3 series fatty acids in the circulating blood. Although this was an open study, patients were specifically asked not to change their usual eating habits and the authors have no evidence to suggest that these instructions were not followed. The reduction in total serum cholesterol over seven years in patients with raised baseline levels was both consistent and significant. However, LDL cholesterol changed little over the first four years of study with the exception of three points, at which a significant increase was observed. After four years the LDL cholesterol was significantly reduced. We are unable to explain the LDL pattern, except to point out that the significant fall at years five, six and seven was accompanied by a significant reduction in total cholesterol. These changes, if confirmed by further studies, could suggest the possibility of an anti-atherogenic role for n-3 fatty acids.

Increases in HDL cholesterol were highly significant at each time point and may be related to the fall in serum triglycerides. The increased HDL-C is suggestive of an enhanced removal of cholesterol, and that a shift in body cholesterol from the serum to the tissue pool is not the mechanism by which the total cholesterol is reduced. The ingestion of n-3 fatty acids appears to lead to a reduction in triglyceride-rich VLDL and, consequently, it appears likely that the synthesis and release of apo A-I from endothelial cells is increased. This probably leads to increased conversion of discoid to spherical HDL due to activating effect of the apoprotein on lecithin:cholesterol acyltransferase (LCAT). It is also possible that an increase in HDL synthesis occurs by stimulation of the end process of HDL formation, allowing for a greater rate of apo A-I incorporation into the final HDL. Although changes in platelet count did not reach significance, about 50% of subjects experienced a reduction in platelet numbers. The incorporation of n-3 fatty acids into the platelet membrane lipids leads to the partial replacement of arachidonic acid in the membrane by EPA which, in turn, leads to the weak aggregating agent, thromboxane A_3, instead of the potent aggregator A_2 (41), thereby reducing the tendency to thrombosis. The study presented here suggests that beneficial changes can be elicited in the concentration of blood lipids. It is interesting that in the long term, both total and LDL cholesterol are reduced significantly. LDL cholesterol was reduced by 0.6 mmol/L after seven years, from an initial level of 4.66 mmol/L. Changes after the end of the study were insignificant. Many patients continued taking MaxEPA, prescribed by their general practitioner, after the end of the study and continued attending our lipid clinic. No significant weight change was observed but it is possible that, despite asking patients not to change diet, the much publicized benefit of using polyunsaturated fats to help lower cholesterol influenced some patients from the mid-1980's. These changes, together with a reduction in the concentration of plasma fibrinogen, may lead to a slowing-down in atheroma formation. Furthermore, a reduction in fibrinogen together with changes induced in thromboxane production demonstrated by other researchers suggests the possibility of a reduction in thrombus formation. Long term safety of MaxEPA consumption in the doses used in this study and assessed by the methods described would appear to suggest no cause for concern.

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The Effect of Beta Blocking Drugs on Lipid Peroxidation in Rat Heart *in Vitro*

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Beta-adrenergic receptor blocking drugs include a structurally related class of drugs that are employed clinically to treat a variety of cardiovascular disorders. Since these drugs exert additional nonspecific effects including membrane stabilization, representative samples including atenolol, dilevolol, labetolol, metoprolol and propranolol were studied to determine their influence on lipid peroxidation. Homogenates or liposomes of adult rat hearts were incubated in the presence of various concentrations of propranolol or equivalent concentrations of dilevolol, labetolol, metoprolol or atenolol. Lipid peroxidation was stimulated with 50 μM $FeSO_4$, 5 μM *t*-butyl hydroperoxide (homogenates) or 0.2 mM citrate $FeSO_4$ (liposomes) plus O_2. Lipid peroxidation, as assessed by both the thiobarbituric acid reaction and chemiluminescence, was reduced in a dose-dependent manner as the propranolol concentration was increased from 1 to 10 mM. The five beta-adrenergic receptor blocking drugs reduced lipid peroxidation both in crude homogenates and in liposomes; their effectiveness was related to their lipophilicity. Dilevolol, propranolol, labetolol and metoprolol at a concentration of 20 mM reduced lipid peroxidation by 45%, 37%, 35% and 28%, respectively. The hydrophilic blocker atenolol was ineffective in reducing lipid peroxidation even at elevated concentrations. Lipophilic beta-blocking drugs apparently are capable of exerting an antioxidant effect in protecting membrane lipids against peroxidation. *Lipids 27,* 539–542 (1992).

Series of clinical trials have recently demonstrated that beta-adrenergic receptor blocking drugs (beta blockers) reduce mortality subsequent to acute myocardial infarction. Beta blockers include a class of structurally related *N*-isopropyl amines. The drugs exert their specific receptor blockade by competing for membrane receptor sites. However beta blockers have also been shown to exhibit a variety of nonspecific effects. For instance, they reduce O_2 consumption and alter the metabolism of the myocardium (1), reduce calcium entry (2), exert local anesthetic activity (3), exert antiarrhythmic action in heart tissue (4) and stabilize membranes (5).

The mechanisms by which the nonspecific effects are exerted are poorly understood. The membrane-stabilizing properties of the drugs may be related to the degree of lipophilicity of certain blockers. The lipophilic characteristic of these compounds seems to be related to their aromatic ring structure. For instance, the *n*-octanol and buffer distribution coefficients range from 20.2 for the propranolol, 11.5 for labetolol, 0.98 for metoprolol and 0.0015 for atenolol (6). Presumably, the greater lipophilicity of propranolol may be due to its naphthalene moiety. In addition, beta blockers are cationic amphiphiles, and that characteristic may enable them to interact with phospholipids (7).

A vast literature implicates oxygen centered radicals as initiators of lipid peroxidation (8,9). The process of lipid peroxidation results in a variety of detrimental changes in cell biology including altered electrophysiological changes that may lead to an increased susceptibility to arrhythmias (10). Since the beneficial effects of beta blockers extend to the central nervous system (5) as well as to the gastric mucosa (11), the beneficial action of the drugs might operate through a mechanism unrelated to membrane receptor blockade. The following experiments were conducted to determine whether beta blockers might protect myocardial tissue by reducing its susceptibility to lipid peroxidation.

MATERIALS AND METHODS

Male Sprague-Dawley-derived rats (200–250 g), originally obtained from Harlan Sprague Dawley (Indianapolis, IN), were housed in standard wire cages and maintained on a 12-h day/12-h night cycle. Rats were given standard rat chow and water *ad libitum.* All animals were sacrificed between 0800–1000 h. The animals were anesthetized with sodium pentobarbital (42 mg/kg), and the hearts were perfused with 100 mL of ice-cold 0.9% NaCl. Hearts were prepared for analysis either as homogenates with 1 g of tissue to 9 mL of 1.15% KCl, or as liposomes. Liposomes were prepared according to the method of Pederson *et al.* (12) by sonicating lipids that had been extracted from heart homogenates according to the method of Folch *et al.* (13). The samples were covered with argon and were maintained in an ice bath until used.

All solutions were made in twice distilled water and passed through Chelex 100 to remove iron (14). Heart tissue susceptibility to lipid peroxidation was determined by incubating aliquots of homogenates for various periods of time either in the presence of a beta blocking drug (at various concentrations) or an equal volume of 0.9% saline. Homogenates received either 100 μL of 50 μM $FeSO_4$, 5 μM *t*-butyl hydroperoxide or an equal volume of H_2O, and were gassed with O_2/CO_2 (95/5%) for 30 s prior to being tightly capped and incubated at 37°C for 1 h. Liposomes were stimulated with 100 μL of 1:1 (vol/vol) citrate Fe^{2+}/citrate Fe^{3+} (0.2 mM). The 0.2 mM citrate Fe solutions were made fresh daily according to the method of Minotti and Aust (15). Thiobarbituric acid reactive substances (TBARS) were assayed by the method of Ohkawa *et al.* (16). Malondialdehyde (MDA) was prepared by the method of Kwon and Watts (17) from 1,1,3,3-tetraethoxypropane (Sigma Chemical Company, St. Louis, MO), and was used as an internal standard.

Ultraweak chemiluminescence was analyzed using a single photon counting technique similar to that of Boveris *et al.* (18). The system consisted of a photomultiplier tube (EMI-GENCOM, 96586, THORN EMI-GENCOM, Fairfield, NJ) housed in a thermoelectric cooler (EMI-GENCOM, Fact 50). The tube output was set through an

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Abbreviations: MDA, malondialdehyde; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

amplifier-discriminator to a counter (EMI-GENCOM, C10) and the data were subsequently transferred to an Apple IIe computer for analysis. The system was calibrated against carbon-14 according to the method of Woodland Hastings and Weber (19). The homogenates or liposomes were placed in a specially constructed 15 × 5 × 50 mm lucite cuvette. During the assay, the 2.5-mL volumes were gently stirred and were continually gassed with O_2/CO_2 (95/5%). Chemiluminescence was stimulated by the addition of $FeSO_4$, citrate Fe or *t*-butyl hydroperoxide as in the TBARS assay. The effect of epinephrine on the TBARS and chemiluminescence assays was measured with epinephrine bitartrate (Sigma Chemical Company). Where appropriate, data are referenced to protein which was determined by the method of Bradford (20).

Values are expressed as mean ± SE. Data were analyzed by analysis of variance followed by the Newman-Keuls test when appropriate. Significance was established at the 0.05 level.

RESULTS

Figures 1 and 2 illustrate a dose-dependent relationship between propranolol, chemiluminescence and TBARS. When propranolol was included in the reaction at a final concentration of 0.01, 0.1, 1 or 10 mM, chemiluminescence and TBARS were significantly reduced relative to the iron stimulated condition. Good agreement was found between the two parameters measured. A regression analysis of the data yielded an r = 0.86.

In order to determine whether the observed inhibitory effect was limited to iron stimulated peroxidation or to propranolol, five beta-blocking agents were tested with peroxidation induced by including *t*-butyl hydroperoxide (5 μM) in the incubation. The data in Figure 3 demonstrate that the drugs dilevolol, propranolol, labetolol, and metoprolol all significantly reduced TBARS relative to the

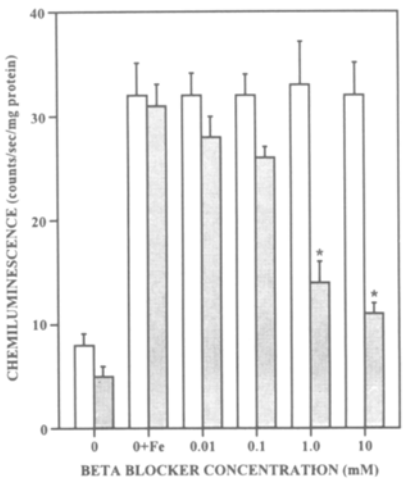

FIG. 1. Inhibition of iron stimulated chemiluminescence from heart homogenates (10% w/v) by mM concentrations of propranolol. Reaction contained 0.5 mL homogenate (10% w/v), 100 μL $FeSO_4$ (50 μM), 5 μL antifoam, 1.795 mL sodium phosphate buffer and 100 μL propranolol or H_2O. Control = 0 with no iron or propranolol, 0 + Fe = iron and no propranol. Data were analyzed by analysis of variance followed by the Newman-Keuls test. Asterisk indicates values significantly different from Fe stimulated values ($P \leq 0.05$).

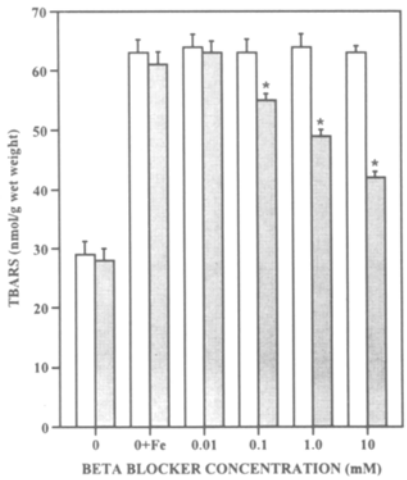

FIG. 2. Inhibition of iron stimulated thiobarbituric acid reactive substance (TBARS) formation from heart homogenates by propranolol. Reaction mixture is as in Figure 1. Data are means ± SE. Asterisk indicates values significantly different from Fe stimulated values ($P \leq 0.05$).

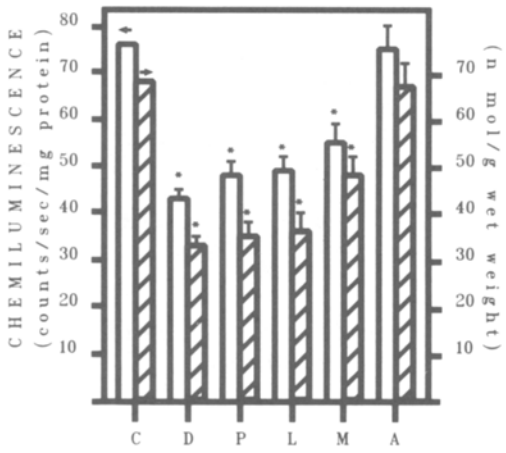

FIG. 3. Inhibition of chemiluminescence (open bars) and thiobarbituric acid reactive substance (TBARS—slash bars) from heart homogenates resultant from incubation with 100 μL of either 20 mM atenolol (A), dilevolol (D), labetolol (L), metoprolol (M) or propranolol (P). Remainder of the reaction mixture is as in Figure 1. Asterisk indicates values significantly different from Fe stimulated values ($P \leq 0.05$).

t-butyl hydroperoxide stimulated condition. Dilevolol which produced a 52% reduction was most effective. Propranolol and labetolol were equally effective in reducing TBARS by 47% and chemiluminescence by 37%. Dilevolol and metoprolol resulted in a 45% and 28% reduction in chemiluminescence, respectively, while atenolol was ineffective in altering TBARS and chemiluminescence. The drugs similarly reduced iron stimulated lipid peroxidation (data not shown).

As the previous data were derived from crude homogenates, additional experiments were conducted to determine whether propranolol would exert a similar effect in lipids extracted from heart homogenates. Figure 4 demonstrates that the drugs' effectiveness in inhibiting lipid

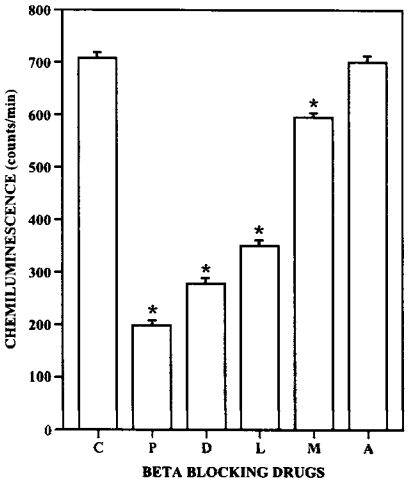

FIG. 4. The effect of 100 μL of either 20 mM atenolol (A), dilevolol (D), labetolol (L), metoprolol (M) or propranolol (P) on liposomal chemiluminescence stimulated by 100 μL of 1:1 (vol/vol) citrate Fe^{2+}/citrate Fe^{3+} (0.2 mM). Remainder of reaction mixture is as in Figure 1. Data are means ± SE. Asterisk indicates significantly different from citrate Fe stimulated control ($P \leq 0.05$).

peroxidation in liposomes was similar to that observed in crude homogenates with the exception that propranolol, rather than dilevolol, was most effective. In addition, Figure 5 illustrates that the inhibitory effect was related to the length of time that liposomes had been preincubated with propranolol prior to stimulating peroxidation by introducing citrate $FeSO_4$ into the reaction.

Propranolol at a final concentration of 10 mM was included with an MDA standard to determine whether propranolol simply interfered with the chemistry of the TBARS formation. Table 1 demonstrates that the treatment failed to reduce TBARS. Furthermore, Table 1 also demonstrates that epinephrine or propranolol added to homogenates at the end of the incubation period did not alter TBARS values.

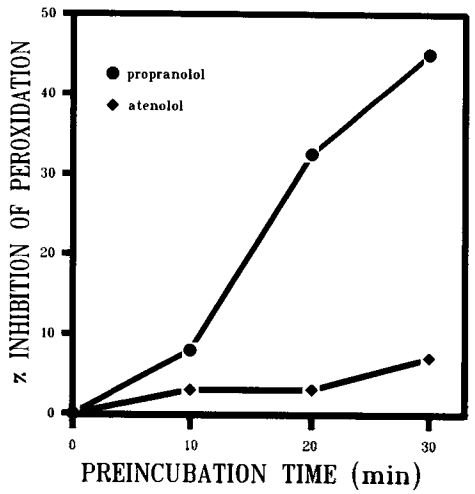

FIG. 5. Relationship between the length of time heart homogenates were preincubated with atenolol or propranolol prior to stimulation with Fe and the % reduction in lipid peroxidation from Fe stimulated controls.

TABLE 1

Influence of Propranolol or Epinephrine on the Thiobarbituric Acid Reaction (TBARS) with Peroxidized Heart Homogenates and Malondialdehyde (MDA) Standards

	Control[a]	Epinephrine[b]	Propranolol
Heart	58.0 ± 2.1[c]	60.3 ± 2.8	59.2 ± 3.1
MDA	0.19 ± 0.1	0.18 ± 0.1	0.21 ± 0.2

[a] Heart control was homogenate stimulated with $FeSO_4$ (as in Fig. 1). MDA control is 6 nmol standard.

[b] Incubation medium contained 10 μg epinephrine per mL or 10 mM propranolol.

[c] Units of measurements are TBARS (nmol/g wet weight) for heart and absorbance for MDA.

DISCUSSION

Meerson and his colleagues (10) were the first to show that inderal (propranolol) resulted in a reduction of catecholamine-induced lipid peroxidation. More recently, beta blockers have been shown to reduce lipid peroxidation of sarcolemma (21,22) and microsomal membranes (23). The present studies confirm the previous findings that the effectiveness of the beta blockers is related to the compounds' lipophilicity (24). Furthermore, our data demonstrate that the inhibition of lipid peroxidation is related to a more general action of the drug and not to a specific receptor blocking mechanism.

Beta-adrenergic blocking drugs have been reported to provide protection against damage in various tissues and conditions. For instance, while there was no benefit from propranolol administration begun 8.5 h post-myocardial infarction (25), the benefits of adrenergic blocker administration prior to tissue insult have been well documented. Several groups have reported that patients who were given beta-blockers prior to surgery suffered less myocardial damage than those taken off blockers prior to surgery (26). Propranolol has also been shown to exert a nonspecific membrane-stabilizing effect on erythrocyte membranes (27). In addition, propranolol administration was associated with a reduction in gastric mucosal damage (11) as well as a reduction in cerebral infarct size in cats subsequent to middle cerebral artery occlusion (28).

Until recently the beneficial influence of beta-adrenergic blocking drugs that could not reasonably be attributed to receptor mediated effects has been explained as nonspecific effects on mitochondrial coupling (29), blood pressure reduction (7) or reduced energy demands (30). A growing body of evidence implicates oxygen centered radical events in membrane injury and malfunction. Barsacchi *et al.* (31) have correlated oxidative stress with decreased contractile function in hearts. Furthermore, pretreatment of hydroperoxide perfused hearts with the radical scavenger butylated hydroxytoluene reduced arrhythmias and the accumulation of malondialdehyde (32).

Arouma *et al.* (23) have recently shown that beta blockers do not act as antioxidants. Beta blockers may exert their non-specific membrane stabilizing effect by reducing lipid peroxidation. Cruickshank and Neil-Dwyer (24) have shown that the beneficial influence of beta-blockers was related to the degree of their lipophilicity. More recently Ondrias *et al.* (33) have confirmed that

eleven beta-blocking drugs interacted with membranes in a structure-dependent manner which was related to their lipophilicity. The partition coefficients for the drugs used in this study were in direct agreement with the order of effectiveness in reducing lipid peroxidation, with the hydrophilic drug atenolol being least effective and dilevolol, the R,R-isomer of labetolol, being the most effective. We interpret the fact that beta-blockers required a preincubation period to be effective as evidence that they exert a lipid peroxidation blocking action. The lipophilic beta-blocking drugs may intercalate into the lipid matrix and reduce the propagation phase of lipid peroxidation in a manner similar to that proposed for tocopherol (34).

ACKNOWLEDGMENTS

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Ascorbate and Phenolic Antioxidant Interactions in Prevention of Liposomal Oxidation

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Efficient prevention of membrane lipid peroxidation by vitamin E (α-tocopherol) may involve its regeneration by vitamin C (ascorbate). Conceivably, the efficacy of antioxidants designed as therapeutic agents could be enhanced if a similar regeneration were favorable; thus, a model membrane system was developed which allowed assessment of interaction of phenolic antioxidants with ascorbate and ascorbyl-6-palmitate. Ascorbate alone (50–200 μM) potentiated oxidation of soybean phosphatidylcholine liposomes by Fe^{2+}/histidine-Fe^{3+}, an effect which was temporally related to reduction of Fe^{3+} generated during oxidation. Addition of 200 μM ascorbate to α-tocopherol-containing liposomes (0.1 mol%) resulted in marked, synergistic protection. Accordingly, in the presence but not absence of ascorbate, α-tocopherol levels were maintained relatively constant during Fe^{2+}/histidine-Fe^{3+} exposure. Probucol (4,4′-[(1-methylethylidine)*bis*(thio)][*bis*[2,6-*bis*(1,1-dimethylethyl)]phenol), an antioxidant which prevents oxidation of low density lipoproteins, and its analogues MDL 27,968 (4,4′-[(1-methylethylidene)*bis*(thio)]-*bis*[2,6-dimethyl]phenol) and MDL 28,881 (2,6-*bis*(1,1-dimethylethyl)-4-[(3,7,11-trimethyldodecyl)thio]phenol) prevented oxidation but exhibited no synergy with ascorbate. Ascorbyl-6-palmitate itself was an effective antioxidant but did not interact synergistically with any of the phenolic antioxidants. Differential scanning calorimetry revealed significant differences among the antioxidants in their effect on the liquid-crystalline phase transition of dipalmitoyl phosphatidylcholine (DPPC) liposomes. Both α-tocopherol and MDL 27,968 significantly reduced the phase transition temperature and the enthalpy of the transition. MDL 28,881 had no effect while probucol was intermediate. The potential for ascorbate or its analogues to interact with phenolic antioxidants to provide a more effective antioxidant system appears to be dictated by structural features and by the location of the antioxidants in the membrane.
Lipids 27, 543–550 (1992).

Vitamin E (α-tocopherol) functions as the major lipophilic antioxidant in biological systems by virtue of its ability to react with lipid peroxyl radicals to terminate the peroxidative process (1). The resultant tocopheroxyl radical can be further oxidized to a number of non-radical products including tocopherol quinone and 8a-(alkyldioxy)tocopherones (2–4). As the loss of a critical cellular defense mechanism by irreversible oxidation would be extremely deleterious to cells, one would expect relatively high cellular levels of α-tocopherol which are accompanied by high turnover rates. However, in most tissues the ratio of α-tocopherol to polyunsaturated fatty acids is on the order of 1:1000 and, furthermore, in certain tissues the biological half-life of α-tocopherol is quite long (5,6). For these reasons, it has been speculated that other cellular reductants interact with partially oxidized α-tocopherol which would, in effect, greatly increase the protective capacity of a cell by enabling a presumably much larger reductant pool to participate in termination reactions.

In vitro studies have suggested that α-tocopherol may be recycled by interaction with glutathione (GSH) or ascorbate. Protection against lipid peroxidation in microsomes (7,8) mitochondria (9) and nuclei (10) has been afforded by GSH, and one possible explanation for the protection was the enzyme catalyzed recycling of α-tocopherol (7). Mitochondria also possess a free radical reductase which uses nicotinamide adenine dinucleotide, reduced (NADH) as a source of reducing equivalents to reduce chromanoxyl radicals (11). Alternatively, in support of an earlier proposal by Tappel (12), direct reduction of the tocopheroxyl radical by ascorbate has been demonstrated (13,14). This regeneration of α-tocopherol by ascorbate has been assumed to be responsible for their observed synergistic protective effect in model lipid peroxidation systems (15,16). However, it has been questioned whether ascorbate can effectively compete with secondary oxidation reactions of the tocopheroxyl radical or whether it reduces another oxidized intermediate such as a tocopherone cation (2).

In the design of antioxidants for treatment of disorders related to membrane lipid oxidation, it may be desirable to prepare compounds which can interact with ascorbate as does α-tocopherol. Many studies have evaluated α-tocopherol analogues in an attempt to define the chemical and structural properties of the molecule which make it ideally suited as a lipophilic peroxyl radical trap (17,18). Moreover, α-tocopherol derivatives have been evaluated for the ability of their respective chromanoxyl radicals to interact with ascorbate (19). In general, as the alkyl groups *ortho* to the phenoxyl radical of the α-tocopherol derivatives increase in size, the second order rate constant for the interaction decreases appreciably, probably due to steric hindrance. These results are of interest as most synthetic antioxidants such as probucol and butylated hydroxytoluene (BHT) are di-*tert*-butyl compounds. *In vivo*, other additional effects such as positioning of the antioxidant in the membrane and reduction of oxidized intermediates other than the tocopheroxyl radical (2) are likely to be of importance. The purpose of this study was to compare the ability of α-tocopherol and three synthetic lipophilic antioxidants to interact synergistically with ascorbate or ascorbyl-6-palmitate in the prevention of iron-induced oxidation of liposomal membranes. Probucol was

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Abbreviations: BHT, butylated hydroxytoluene; DPPC, dipalmitoyl phosphatidylcholine; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; MDL 27,968, 4,4′-[(1-methylethylidene)*bis*(thio)]*bis*[2,6-dimethyl]-phenol; MDL 28,881, 2,6-*bis*(1,1-dimethylethyl)-4-[3,7,11-trimethyldodecyl)thio]phenol; NADH, nicotinamide adenine dinucleotide, reduced; probucol, 4,4′[(1-methylethylidene)*bis*(thio)]*bis*[2,6-*bis*(1,1-dimethylethyl)]phenol; TBARS, thiobarbituric acid reactive substances; T_m, phase transition temperatures.

evaluated as it is an effective antioxidant in low density lipoproteins (20) and prevents atherosclerosis in animal models (21–23). To address the issue of *ortho* substitution, MDL 27,968 was chosen as it contains methyl, rather than di-*tert*-butyl, groups. MDL 28,881 has a similar substitution at the *ortho*-position but contains an acyl chain similar to α-tocopherol which may allow assessment of membrane positioning as a determinant of synergistic interaction with ascorbate.

MATERIALS AND METHODS

Probucol (4,4′-[(1-methylethylidene)*bis*(thio)]*bis*[2,6-*bis*-(1,1-dimethylethyl)]phenol) and its analogues MDL 28,881 (2,6-*bis*(1,1-dimethylethyl)-4-[(3,7,11-trimethyldodecyl)-thio]phenol) and MDL 27;968 (4,4′-[(1-methylethylidene)-*bis*(thio)]*bis*[2,6-dimethyl]phenol) were synthesized by Marion Merrell Dow (Cincinnati, OH) and the Dow Chemical Company (Midland, MI), respectively (24). The structures of these 3 compounds and α-tocopherol are shown in Figure 1. The α-tocopherol and δ-tocopherol were obtained from Kodak (Rochester, NY). Soybean phosphatidylcholine (Type III-S), Ferrozine, ferrous chloride, ferric chloride, ethylenediaminetetraacetic acid (EDTA), histidine, trichloroacetic acid, butylated hydroxytoluene, ascorbic acid, ascorbyl-6-palmitate, and thiobarbituric acid were purchased from Sigma (St. Louis, MO). Dipalmitoyl phosphatidylcholine (DPPC) was a product of Avanti Polar Lipids (Birmingham, AL). All other chemicals were obtained from commercial sources and were of the highest grade available.

Liposome preparation and oxidation. Liposomes comprised primarily of single bilayers were prepared by ethanol injection essentially as described by Kremer *et al.* (25). Soybean phosphatidylcholine and, where indicated, the phenolic antioxidants or ascorbyl-6-palmitate were dried under a stream of N_2. Typically, the material was reconstituted in 100 μL of ethanol and injected using a Hamilton syringe (Reno, NV) into 10 mL of 10 mM Tris/50 mM NaCl, pH 7.0 at 37°C while mixing. The final concentration of the phosphatidylcholine was 0.5 mM and the antioxidants included at various mol% relative to the phospholipid concentration. The final lipid concentration is 0.5 mM, thus for all antioxidants 0.1 mol% is equivalent to 0.5 μM. Incubations were carried out at 37°C in 25 mL beakers under an air atmosphere in a metabolic shaking incubator. Fe^{2+} was prepared in ice-cold, nitrogen-purged, doubly distilled, deionized water and kept on ice to minimize autoxidation. The histidine-Fe^{3+} was prepared as a stock solution (25 mM histidine/5 mM $FeCl_3$) in 10 mM Tris/50 mM NaCl and the pH adjusted to approximately 6.5. Above this pH, the complex began to dissociate as evidenced by Fe^{3+} precipitation. When added to the incubation mixture at pH 7.0, no precipitate was evident. Where appropriate, ascorbate was added to the incubations just prior to initiation of oxidation with Fe^{2+} (50 μM) and histidine-Fe^{3+} (250 μM histidine/50 μM Fe^{3+}). An assessment of oxidation was achieved by measurement of thiobarbituric acid reactive substances (TBARS). At the indicated times, a 1-mL aliquot of the incubation mixture was removed and added to a tube containing 2 mL of 0.67% thiobarbituric acid in 0.25 N HCl and 10% trichloroacetic acid (2:1, vol/vol) and 0.033% BHT (26). The samples were heated at 100°C for 20 min and centrifuged after cooling. The absorbance of the supernatant at A_{532} minus A_{580} to account for any turbidity was read, and TBARS were quantitated by comparison to a standard curve of malondialdehyde equivalents generated by acid catalyzed hydrolysis of 1,1,3,3-tetraethoxypropane. Each experiment was repeated at least twice with differences in TBARS formation between duplicate samples generally less than 10%.

Iron oxidation/reduction. The concentration of Fe^{2+} in the incubations was determined by removing 0.9 mL and adding to 0.1 mL of 10 mM ferrozine [3-(2-pyridyl)-5,6-*bis*(4-phenyl-sulfonic acid)-1,2,4-triazine]. The absorbance of the ferrozine/Fe^{2+} complex was read at 564 nm and compared to a standard curve prepared from known amounts of Fe^{2+}. The reduction of histidine-Fe^{3+} by ascorbate was assessed by incubating histidine-Fe^{3+} (500 μM histidine/100 μM Fe^{3+}) with 200 μM ascorbate for 0–15 min. Aliquots (0.9 mL) of the reaction mixture were taken at various times and analyzed for Fe^{2+} content.

α-Tocopherol determination. The content of α-tocopherol in liposomes was determined by high-performance liquid chromatography using fluorometric detection (5) with some minor modification (27). One-mL aliquots of liposomes containing 0.1 mol% α-tocopherol were removed at various intervals and added to 1 mL of 0.1 M sodium dodecylsulfate. δ-Tocopherol (500 pmoles) in ethanol was added as an internal standard followed by 2 mL of ethanol and vigorous mixing. The tocopherols were extracted into 2 mL of heptane and the tubes centrifuged at low speed for 5 min. The heptane layer was removed, dried under N_2, and the material reconstituted in methanol. Chromatography was performed using an LKB (Bromma, Sweden) 2150 pump, a Waters (Milford, MA) 712 wisp autoinjector and a McPherson (Acton, MA) FL-749 spectrofluorometer. The mobile phase consisted of methanol/water (98:2, vol/vol) and was run at a flow rate of 0.75 mL/min. A Shodex (Waters, Milford, MA) ODSpak C18 reverse phase column (15 cm × 4.6 mm, 5 μm) was used and fluorometric detection performed with excitation at 210 nm and

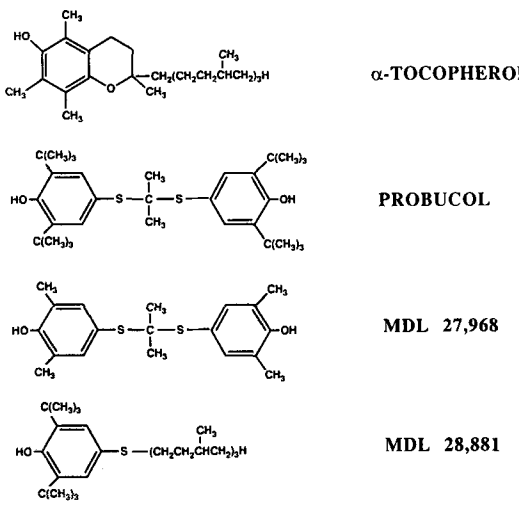

FIG. 1. Structures of α-tocopherol and other phenolic antioxidants.

emission monitored using a 270 nm cut-off filter. The retention time for δ-tocopherol was approximately 9 min and 10.5 min for α-tocopherol with a detection limit of approximately 50 pmol. Quantitation of α-tocopherol was by comparison to the peak area of δ-tocopherol. All extractions were done on ice and in capped tubes to minimize oxidation of the tocopherols.

Differential scanning calorimetry (DSC). Mixtures of DPPC and various mole fractions of antioxidants were prepared by mixing the lipid and antioxidant in chloroform/methanol (1:1, vol/vol), drying under N_2 and vacuum, and swelling in 0.15 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, 0.1% sodium azide, pH 7.4 for 1 h at ~50°C. The final concentration of DPPC was 0.5 mg/mL. DSC was performed on a Microcal MC-2 differential scanning calorimeter (Northampton, MA) at a scan rate of 60°C/h. DPPC alone was scanned at 20°C/h. The differential voltage signal from the thermopiles, the temperature of the heat sink and the time were recorded at 10 s intervals on an IBM-PC-AT computer with a PASCAL program written by MIRAC (Cincinnati, OH). The data were converted to cal/g°C after dividing by the scan rate and the weight of sample. Enthalpies were calculated by numerical integration; the phase transition temperatures (T_m) correspond to the temperatures at which the heat capacities reach maximum values during the transitions.

RESULTS

Incubation of soybean phosphatidylcholine liposomes with Fe^{2+}/histidine-Fe^{3+} resulted in immediate oxidation as has been reported for other Fe^{2+}/Fe^{3+} mixtures (Fig. 2) (28). Following the initial 2 min time period, TBARS formation increased relatively slowly. Histidine-Fe^{3+} was utilized as ascorbate can influence the Fe^{2+}/Fe^{3+} redox balance and thereby inhibit lipid peroxidation independent of a recycling α-tocopherol. Ascorbate is apparently incapable of reducing histidine-Fe^{3+} (29) and, in agreement, we observed no ascorbate oxidation with histidine-Fe^{3+} and no subsequent appearance of Fe^{2+} (data not shown).

The incorporation of increasing amounts of α-tocopherol in the liposomes led to a successive delay in the onset of oxidation which eventually proceeded at a rate similar to that observed in the first two minutes of incubation in the absence of α-tocopherol (Fig. 2A). The addition of ascorbate (50–200 μM) resulted in a slight inhibition of oxidation at 2 min, yet the rate of oxidation remained relatively constant leading to an overall increase in TBARS formation relative to that observed in the absence of ascorbate (Fig. 2B). This is noteworthy as the potential exists for ascorbate to interact with the initiating species or, at high concentrations, to directly terminate peroxidation which could lead to the erroneous conclusion that ascorbate was recycling α-tocopherol. The stimulation by ascorbate in this model system is likely explained by the data shown in Figure 3. In the absence of liposomes Fe^{2+} autoxidation was minimal in our buffer system but the addition of liposomes to Fe^{2+}/histidine-Fe^{3+} led to complete oxidation of the Fe^{2+} within 2 min. Thus, no reducing equivalents remained to propagate the peroxidative process. Inclusion of ascorbate (50–200 μM) delayed depletion of a pool of Fe^{2+} for greater than 10 min which agrees with the continued rise in TBARS formation.

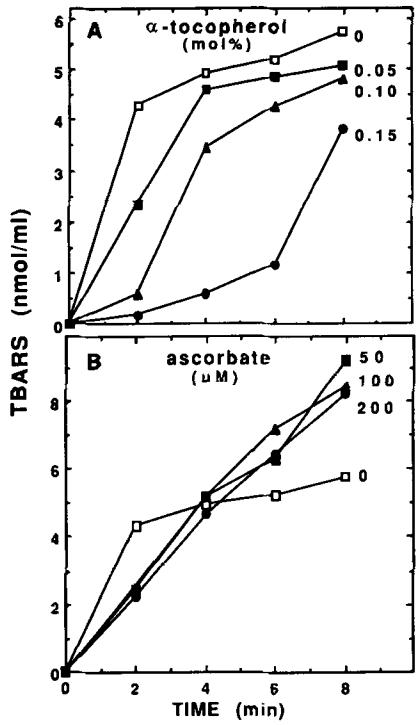

FIG. 2. Effect of varying α-tocopherol or ascorbate concentrations on liposomal peroxidation. Liposomes (0.5 mM) were oxidized with Fe^{2+}/histidine-Fe^{3+} in the presence of α-tocopherol (0–0.15 mol%; Panel A) or ascorbate (0–200 μM; Panel B). Aliquots were removed at various timepoints and analyzed for TBARS as described in detail in Materials and Methods. Data points represent the averages of 2–4 separate incubations.

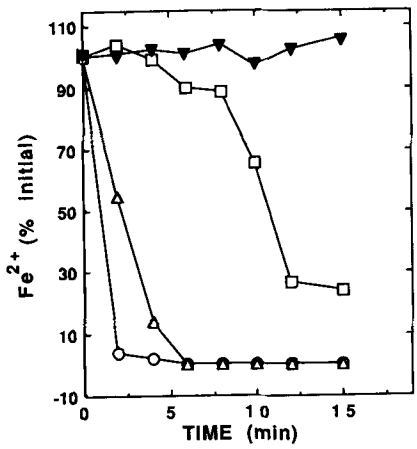

FIG. 3. Effect of ascorbate or α-tocopherol on Fe^{2+} concentrations in liposomes oxidized with Fe^{2+}/histidine-Fe^{3+}. Fe^{2+} concentrations were determined in the absence of liposomes (▼) or with liposomes and Fe^{2+}/histidine-Fe^{3+} (○) in the presence of 200 μM ascorbate (□), or 0.1 mol% α-tocopherol (△). The average initial concentration of Fe^{2+} for all incubations was 52.9 ± 2.2 μM (mean ± SE). Determinations at each timepoint were done in duplicate.

Similarly, the lag in TBARS formation induced by 0.1 mol% α-tocopherol was reflected in a slightly slower rate of Fe^{2+} oxidation. As ascorbate does not reduce histidine-Fe^{3+}, these results suggest that ascorbate can

reduce Fe^{3+} generated by Fe^{2+} oxidation. The net effect is to continue to provide a source of reducing equivalents which catalyzes peroxidative reactions.

For each of the four phenolic antioxidants, a series of concentration curves were obtained such as that shown for α-tocopherol in Figure 2A. From these curves a concentration of each antioxidant was chosen such that peroxidation was delayed only slightly. This allowed for a clearer distinction between synergistic, or merely additive effects, when interaction with ascorbate was evaluated. As observed in Figure 4, 0.4 mol% was the concentration chosen for MDL 27,968 and MDL 28,881 while probucol was 0.3 mol% and α-tocopherol was 0.1 mol%. The presence of 200 μM ascorbate led to a significant increase in TBARS in the absence of a phenolic antioxidant, but a complete suppression of oxidation in the presence of 0.1 mol% α-tocopherol (Fig. 4A). With MDL 27,968 and probucol, the presence of ascorbate led to only a slight lag period before peroxidation began which again reached a level greater than twice that observed in the absence of ascorbate (Fig. 4B and 4C). In the liposomes containing 0.4 mol% MDL 28,881, a lag period of 6 min was observed with ascorbate, at which point peroxidation increased in a linear fashion to eventually exceed that of the control incubation (Fig. 4D).

The lag period preceding the onset of oxidation is characteristic of chain terminating antioxidants and correlates with oxidation of the antioxidant (15). This finding is corroborated by the results in Figure 5A which demonstrate that, in the absence of ascorbate, a marked increase in TBARS formation correlated with the loss of α-tocopherol. When ascorbate was included in the incubation containing 0.1 mol% α-tocopherol, the levels of α-tocopherol remained relatively constant over the 15 min time period and, accordingly, no TBARS formation was observed.

The lack of interaction of antioxidants other than α-tocopherol with ascorbate may be due to differences in interaction with and orientation in the liposomes. An evaluation of the interaction of the antioxidants with phospholipids was performed by examining their effect on the liquid-crystalline phase transition of DPPC using differential scanning calorimetry. As shown in Figure 6, probucol, α-tocopherol and MDL 27,968 broaden T_m of DPPC with α-tocopherol having the most dramatic effect on the enthalpy of the transition (Fig. 7A). Both α-tocopherol and MDL 27,968 reduced T_m approximately 4°C at a concentration of 20 mol% (Fig. 7B). In contrast, MDL 28,881 had relatively little effect on either T_m or the enthalpy of the transition.

The inability of ascorbate to provide synergistic protection with antioxidants other than α-tocopherol could also be related to physical constraints imposed by compartmentation of ascorbate in the aqueous phase and the antioxidant within the hydrophobic membrane region. Therefore, the effect of a lipophilic ascorbate analogue, ascorbyl-6-palmitate, was tested in the liposomal system. As demonstrated in Figure 8, ascorbyl-6-palmitate itself provides good protection against iron-induced oxidation which limited the concentration which could be tested in synergy experiments to 0.25 or 0.5 mol%. With all four phenolic antioxidants, the addition of ascorbyl-6-palmitate resulted in the induction of only a slight lag period (Fig. 9).

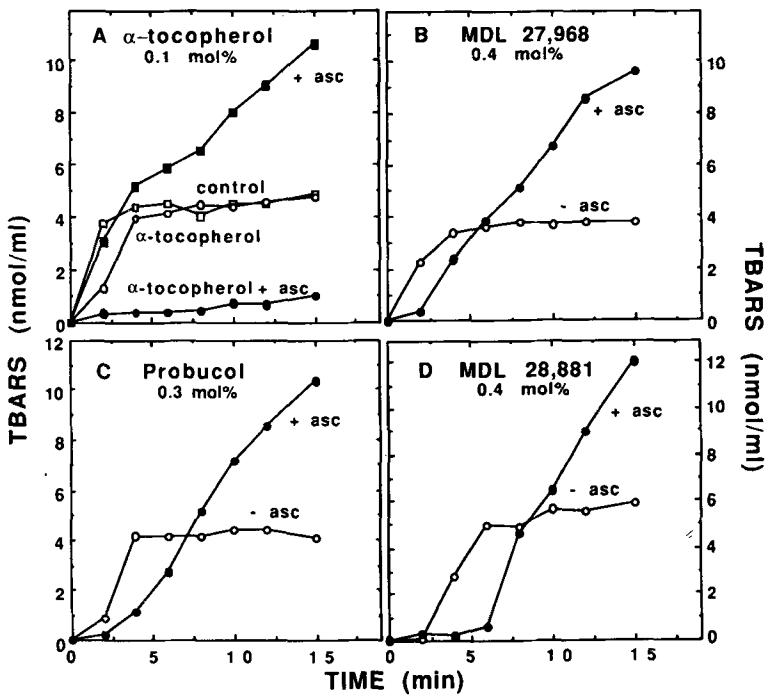

FIG. 4. Effect of ascorbate on peroxidation of phenolic antioxidant-containing liposomes. Liposomes containing the phenolic antioxidants were oxidized with Fe^{2+}/histidine-Fe^{3+} in the absence (open symbols) or presence (closed symbols) of 200 μM ascorbate. Incubations were sampled at various timepoints for TBARS as detailed in Materials and Methods. Values are the average of two incubations. In Panel A, control incubations without α-tocopherol are included (open squares) and with ascorbate (closed squares).

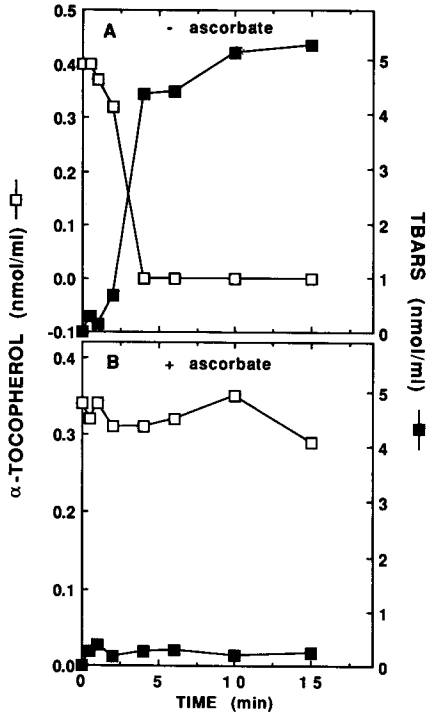

FIG. 5. α-Tocopherol and TBARS concentrations in liposomes undergoing peroxidation in the presence or absence of ascorbate. Liposomes contained 0.1 mol% α-tocopherol and, either no (panel A) or 200 μM (panel B) ascorbate. At the indicated time, the reaction mixtures were sampled for the determination of α-tocopherol (□) and TBARS (■) as outlined in Materials and Methods. Each data point represents the average of two separate incubations for both α-tocopherol and TBARS with values varying less than 10%.

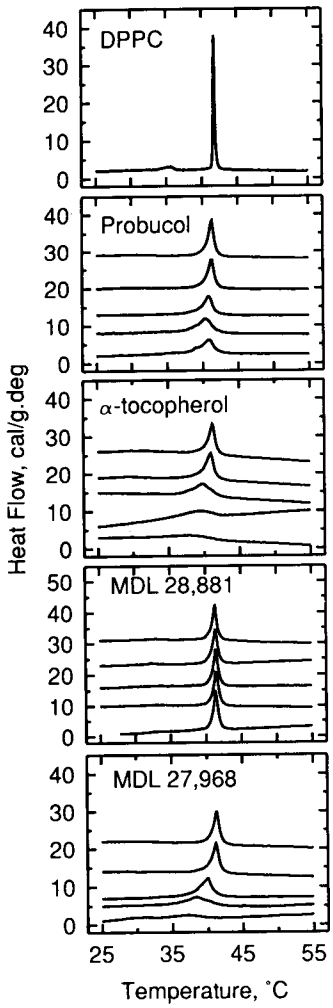

FIG. 6. DSC of mixtures of antioxidants with DPPC. Antioxidants were mixed with DPPC and prepared as liposomes at mol fractions of 1, 2, 5, 10 and 20 mol%. The scans are in order from top to bottom with increasing concentrations of antioxidants. Two separate DPPC/antioxidant mixtures were prepared and scanned for each concentration.

DISCUSSION

The present study confirms the extremely effective interaction between ascorbate and α-tocopherol in prevention of lipid oxidation (15,30). In the presence of very low amounts of α-tocopherol, ascorbate totally prevented oxidation while in the absence of α-tocopherol it greatly potentiated overall TBARS production. This is a dramatic illustration of the potent antioxidant capacity of these agents acting in concert to not only inhibit oxidation induced by Fe^{2+}/histidine-Fe^{3+}, but to completely counteract the significant enhancement of peroxidation induced by the ability of ascorbate to maintain Fe^{2+} levels. In the absence of ascorbate, Fe^{2+}/histidine-Fe^{3+} treatment led to a complete loss of α-tocopherol within the first few min which was accompanied by TBARS formation (Fig. 5). In the presence of ascorbate, α-tocopherol levels remained relatively constant throughout the 15 min of incubation and no significant oxidation of the liposomes was noted. Importantly, the use of Fe^{2+}/histidine-Fe^{3+} as an initiating system minimizes the potential for ascorbate, at the concentrations used herein, to exert antioxidant effects independent of recycling of α-tocopherol.

In contrast to α-tocopherol, the other three phenolic antioxidants did not interact synergistically with ascorbate. Both probucol and MDL 28,881 have *tert*-butyl substituents in the *ortho* positions which may present steric hindrance to the ascorbate (19). MDL 27,968 is similar to probucol except that methyl, rather than *tert*-butyl groups, are the *ortho* substituents yet there was still no apparent interaction. There are several possible explanations for this finding. One is that MDL 27,968 is situated in the membrane such that a phenoxyl radical is not oriented to the aqueous side. This may result from the lack of a phytyl tail which has been suggested to align α-tocopherol parallel with fatty acyl chains of the bilayer, thus orienting the chromanol ring toward the outer regions (reviewed in ref. 31). The similarity in the effects of α-tocopherol and MDL 27,968 on the T_m of DPPC suggests that MDL 27,968 also orients itself in the membrane but its lack of a hydrocarbon chain may cause it to be buried within the membrane, thus limiting access to ascorbate. Such differences may be indicated by the difference in the enthalpy of transition for α-tocopherol relative to MDL 27,968. Precedence for such an effect was provided by Takahashi *et al.* (32) who demonstrated that the rate of interaction between ascorbate and spin probes decreased as the nitroxide radical was increasingly buried in the bilayer.

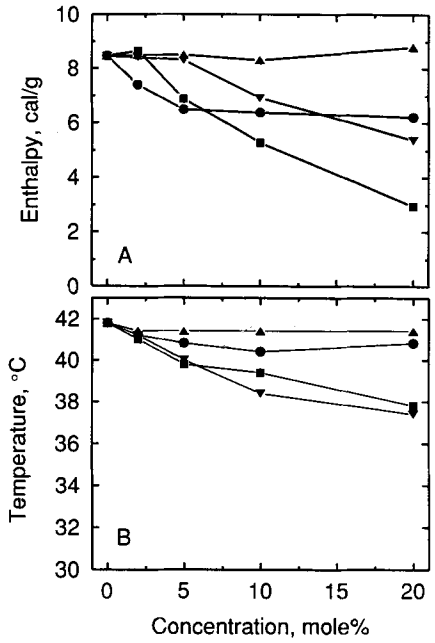

FIG. 7. Enthalpies and temperatures of the liquid-crystalline phase transition of DPPC liposomes doped with antioxidants. Enthalpies (A) and temperatures (B) were calculated for liposomes comprised of DPPC and the various antioxidants. (●) Probucol, (■) α-tocopherol, (▲) MDL 28,881 and (▼) MDL 27,968.

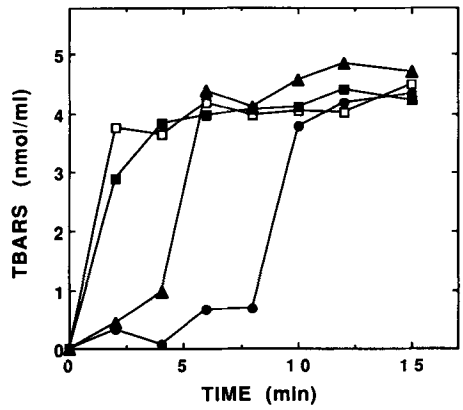

FIG. 8. Influence of ascorbyl-6-palmitate on TBARS formation. Liposomes containing various concentrations of ascorbyl-6-palmitate were treated with Fe^{2+}/histidine-Fe^{3+} and sampled at various times for TBARS. The concentrations (mol%) of ascorbyl-6-palmitate were (□) 0, (■) 0.25, (▲) 0.5 and (●) 0.75. Values represent the average of 2-3 determinations.

MDL 28,881 contains a phytyl tail as does α-tocopherol but does not interact well with phospholipids. One interpretation of the data is that the bulkiness of the *tert*-butyl groups causes the ring portion of MDL 28,881 to reside outside the membrane and thus have less of an effect on

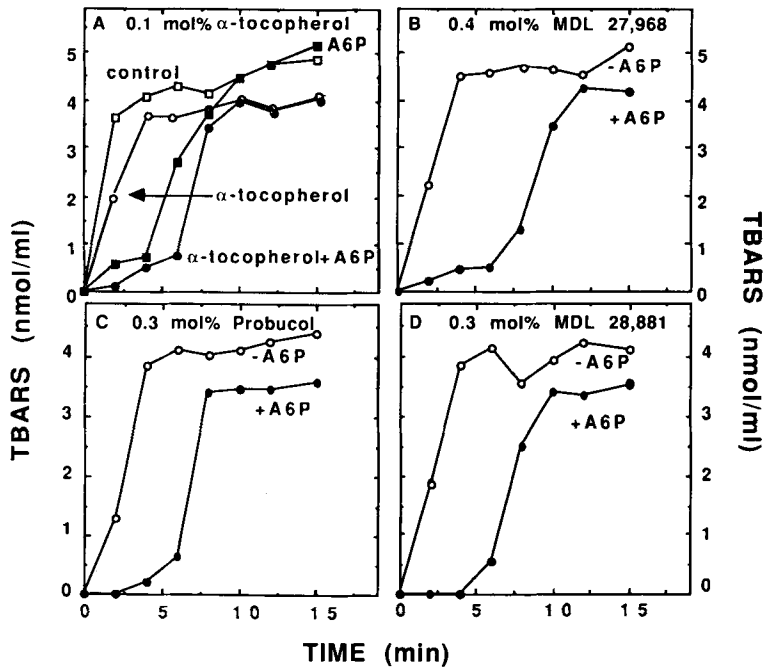

FIG. 9. Effect of ascorbyl-6-palmitate on peroxidation of phenolic antioxidant-containing liposomes. Phenolic-antioxidant containing liposomes were oxidized with Fe^{2+}/histidine-Fe^{3+} in the absence (open symbols) or presence (closed symbols) of 0.25 mol% ascorbyl-6-palmitate. Aliquots were taken at the indicated times for analysis of TBARS as described in Materials and Methods. Data points represent the mean of at least two incubations. In Panel A, control incubations without α-tocopherol are included (open squares) and with ascorbate (closed squares).

the DPPC. We have observed similar DSC scans with MDL 73,335, a tocopherol analogue in which the acyl chain has been replaced with an ethyl amino group (33) which considerably decreases its interaction with DPPC (Thomas, C.E., McLean, L.R., Parker, R.A., and Ohlweiler, D.F., unpublished observations). This would render the phenoxyl radical of MDL 28,881 accessible to ascorbate, and there did appear to be a slight, greater than additive effect when the antioxidants were combined (Fig. 4D).

Another explanation is suggested by the studies of Burton and co-workers (17) which demonstrated that positioning of the pair of pi electrons of the oxy substituent *para* to the hydroxyl group in an orientation perpendicular to the plane of the ring imparts stability to the phenoxyl radical. Such structural stability is lacking in MDL 27,968 and, additionally, it does not have the *ortho-tert*-butyl substituents which greatly stabilize the phenoxyl radicals of probucol and MDL 28,881. The corresponding decrease in the half-life of the phenoxyl radical of MDL 27,968 would lessen the likelihood of its reduction by ascorbate.

The possibility that the phenolic antioxidants other than α-tocopherol may orient in the membrane in a manner which precludes access to ascorbate led us to evaluate a lipophilic derivative of ascorbate, ascorbyl-6-palmitate, for possible interaction with these antioxidants. We observed that ascorbyl-6-palmitate itself was a relatively potent antioxidant (Fig. 8), in agreement with Pryor *et al.* (34) who studied its effect on O_2 uptake in micelles. However, when added to phenolic antioxidant-containing liposomes, no dramatic, synergistic interactions were observed (Fig. 9). The lack of synergy with α-tocopherol can be rationalized by the orientation of its phenoxyl radical toward the aqueous environment. The absence of synergy with probucol and MDL 28,881 may again indicate that bulky groups as *ortho* substituents prevent reduction of the phenoxyl radical as reported for 6-*O*-steroyl-ascorbate (19). On the other hand, Kagan *et al.* (35) recently reported that ascorbyl palmitate could reduce the phenoxyl radical of BHT and related analogues as determined by electron spin resonance spectroscopy. Clearly, physical accessibility of the reductant to the phenoxyl radical is a critical determinant of synergistic antioxidant activity in a membrane system.

It must also be considered that the lack of synergy could be due to an insufficient amount of ascorbyl-6-palmitate. However, several lines of evidence argue against this. First, the concentration of ascorbate required to totally prevent oxidation in the presence of α-tocopherol was much lower than the 50–200 μM used in these studies. The actual ascorbate concentrations required could not be determined as they were below the limit of detection of our assay which was approximately 14 μM. Secondly, in the absence of ascorbate, 0.2–0.3 mol% of α-tocopherol provided complete protection against oxidation. With 0.25 or 0.5 mol% ascorbyl-6-palmitate (1.25 or 2.5 μM), and with 0.1 mol% α-tocopherol, we observed no synergistic interaction. Thus, if even only 20–40% of the added ascorbyl-6-palmitate reacted with the α-tocopherol, this should have provided the same results as 0.2–0.3 mol% α-tocopherol and totally prevented oxidation. Together these findings suggest that in liposomal membranes interaction between ascorbyl-6-palmitate and α-tocopherol is restricted, probably due to physical inaccessibility.

It is clear from these studies that ascorbate and α-tocopherol interact synergistically to prevent iron-induced liposomal oxidation. Importantly, oxidation of liposomes with Fe^{2+}/histidine-Fe^{3+} minimizes the possibility that ascorbate is directly interacting with other radicals to spare the α-tocopherol as ascorbate actually stimulates peroxidation by reducing the Fe^{3+} generated during peroxidation. It cannot be determined, however, whether ascorbate recycles α-tocopherol from the tocopheroxyl radical or from 8a-(alkyldioxy)-tocopherones which constitute approximately half of the α-tocopherol oxidized by peroxyl radicals in liposomes (4). Nonetheless, this system may provide a facile means by which to evaluate synthetic antioxidants for synergistic interaction with ascorbate in a model which resembles biological membranes and may have more relevance than studies conducted in solution (19,36). However, evaluation of ascorbate reduction of phenoxyl radicals as studied by electron spin resonance spectroscopy would provide some useful information regarding the effect of *ortho*-substitution and complement the peroxidation studies described herein. The three synthetic antioxidants tested to date did not interact synergistically with ascorbate or ascorbyl-6-palmitate, a phenomenon which appears attributable to their structural and physical characteristics. Further experimentation will be required to determine the criteria which allow recycling of phenolic antioxidants by ascorbate and/or analogues thereof.

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^{31}P NMR of Tissue Phospholipids: Competition for Mg^{2+}, Ca^{2+}, Na^+ and K^+ Cations

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Phosphatidylcholine (PC), phosphatidylethanolamine (PE), ethanolamine plasmalogen (EPLAS), sphingomyelin (SPH), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), phosphatidylglycerol (PG) and phosphatidic acid (PA) were dispersed together in Cs(ethylenedinitrilo)tetraacetic acid-scrubbed chloroform/methanol solution, and high resolution ^{31}P nuclear magnetic resonance spectra were recorded. In separate titration experiments, Mg^{2+} and Ca^{2+} were added to the dispersed phospholipid mixture to determine the relative interaction potentials of each of the phospholipids for each of the added cations. The association of cations with individual phospholipids was indicated by ^{31}P chemical-shift changes, signal broadening, signal quenching or a combination of these. The titrations revealed that CL had the highest, and PA the next highest, interaction potential for Mg^{2+} cations. In contrast, PS and PA had the highest, and CL the next highest, interaction potential for Ca^{2+}. Considering only interactions with Ca^{2+} ions, the phospholipids can be divided into three distinct groups: PS and PA (high interaction potential); CL, PI and PG (intermediate interaction potential); and EPLAS, PE, SPH and PC (essentially no interaction potential). The two phospholipids with the least interaction potential for either of the alkaline-earth cations were PC and SPH. Na^+ and K^+ ion interactions with PA, CL, PI and PG were unique and resulted in positive chemical-shift changes relative to the chemical shifts in the presence of Cs^+ ions. Relative to both Cs^+ and K^+ ions, chemical shifts in the presence of Na^+ ions were deshielded $\delta >$ 0.1 ppm in the order PA > CL > PI > PG.

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Investigations have attempted to gauge the nature of interactions between membrane phospholipids and inorganic cations, and particularly Ca^{2+} (1-10). From these studies differing conclusions have been derived, as data interpretation is complicated because of the complexity of the lamellar, vesicular or micellar structures that phospholipids produce in aqueous media (6). Moreover, the procedures that have been employed to probe the interactions of phospholipids with cations either in aqueous media or organic solvents, have limited such analyses to the examination of one or two phospholipids at a time.

Observation of phospholipids by ^{31}P nuclear magnetic resonance (NMR) spectroscopy in a hydrated chloroform/methanol medium circumvents many of these analytical limitations (9,11). Using this approach, more than 15 different phospholipid functional groups have been monitored simultaneously, thus permitting competitive binding to be monitored between phospholipid polar head groups and inorganic cations (9).

^{31}P NMR recognizes interactions of phospholipids with cations depending on the type of complex formed, on the nuclear properties of the cation and on the chemical equilibria that exist. The signal from a phosphate interacting with a cation will undergo a chemical-shift change that may be either positive or negative (9,12,13). The signal may typically broaden due to shorter ^{31}P NMR relaxation times (9,14-16). The signal may be quenched, that is, extensively broadened; such broadened ^{31}P NMR signals can be detected by wide-line spectroscopic methods (6).

In the present study, mixtures of approximately equivalent amounts of phospholipids in solution were allowed to interact with a fractional equivalent of a single inorganic cation and were monitored by ^{31}P NMR. The hypothesis was that the cation will interact with the phospholipid polar head group with which it has the highest interaction potential. Should two or more polar head groups exhibit nearly equivalent interaction potentials for a given cation, the cation will be partitioned between these groups, with the remaining phospholipids being essentially non-interactive, *i.e.*, unaffected chemically and spectroscopically.

Phosphatidylcholine (PC), choline plasmalogen (CPLAS), phosphatidylethanolamine (PE), ethanolamine plasmalogen (EPLAS), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), cardiolipin (CL), sphingomyelin (SPH), phosphatidic acid (PA) and an unidentified phospholipid isolated from human lens (U) (17), were selected for this interaction study. Mg^{2+}, Ca^{2+}, Na^+, K^+ and Cs^+ were the cations selected. Similar studies involving interactions with Al^{3+} have already been carried out (18).

METHODS

Phospholipid preparations of high purity were obtained from Sigma Chemical Co. (St. Louis, MO), P-L Biochemicals, Inc. (Milwaukee, WI) and Life-Science Resources (Milwaukee, WI).

Phospholipid-alkaline-earth-ion titrations. From stock solutions of specific phospholipids in chloroform, approximately 4 μmole each of PC, PE, EPLAS, PS, SPH, PI, CL, PG and PA were combined in a total volume of 3 mL of chloroform containing 20% benzene-d_6 (Reagent A). To this solution, 1 mL of methanol containing 20% aqueous 0.2 M Cs(ethylenedinitrilo)tetraacetic acid (EDTA), pH 6, was added (Reagent B). The mixture was stirred, transferred to a 10-mm NMR tube, and allowed to separate into

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Abbreviations: CL, cardiolipin; CPLAS, choline plasmalogen; EDTA, (ethylenedinitrilo)tetraacetic acid; EPLAS, ethanolamine plasmalogen; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; U, unidentified phospholipid.

two phases, an organic phase and an upper aqueous phase (9). A Teflon™ NMR vortex plug was inserted into the NMR tube so that the aqueous phase was slowly extruded through the breather port of the vortex plug and could be aspirated from the tube. The aqueous phase (containing EDTA) was removed as completely as possible, to minimize residual EDTA contamination of the sample. Prior to adding metal ions, a ^{31}P NMR spectrum was recorded to measure the relative proportions of the phospholipids present (Fig. 1). The cations were introduced into the sample after withdrawing the vortex plug by injecting 1–10 μmoles of 1M aqueous cation solution into the chloroform phase by microliter syringe. The vortex plug was then partially re-inserted into the tube, and the sample was mixed by tipping the tube several times. Subsequently, the vortex plug was repositioned for NMR analysis. During the titrations, a minimal aqueous phase was generated by the addition of the aqueous alkaline-earth chloride solutions which accumulated above the chloroform layer; this aqueous phase was allowed to remain in contact with the chloroform phase throughout the titration, so that equilibria were maintained. However, only the organic phase was visible to the receiver coil.

A series of ion concentrations was measured so that each phospholipid resonance could be followed throughout its course. When signal congestion occurred, computerized deconvolution and integration routines were used to aid in signal analysis (17).

The stability of pH and the stability with time of the measured NMR parameters, including chemical shifts, signal widths and integrated signal areas, were taken as evidence that chemical equilibration had been achieved (19). Upon incremental cation addition, the samples were mixed thoroughly for several minutes to allow for a fine dispersion of the aqueous phase in the organic phase. Final NMR data were recorded, usually within 1 min, when no further changes with time were detectable. The time interval between sample mixing and NMR measurements, however, was nominally greater than 10 min.

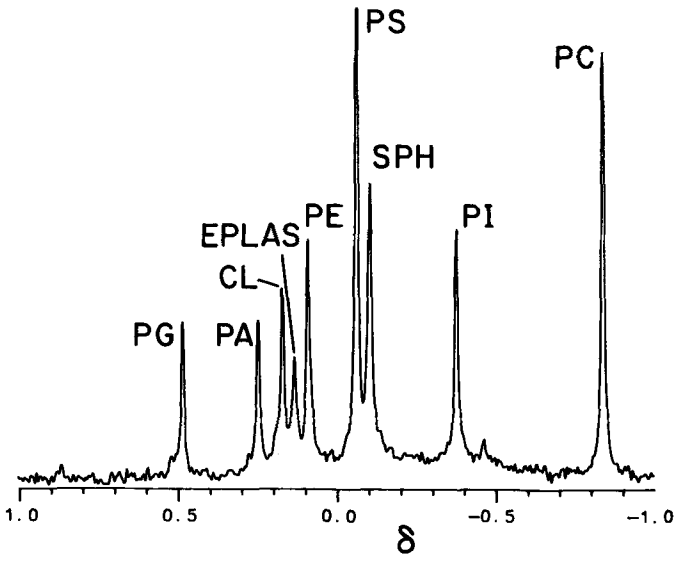

FIG. 1. ^{31}P NMR spectrum of a total rat heart lipid extract in which the endogenous signals of PG and PA have been enhanced by the addition of the pure phospholipids: PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin; EPLAS, ethanolamine plasmalogen; PE, phosphatidylethanolamine; PS, phosphatidylserine; SPH, sphingomyelin; PI, phosphatidylinositol; and PC, phosphatidylcholine.

The benzene-d_6 in the chloroform phase provided the internal NMR reference for field-frequency stabilization (9). The initial phospholipid profile was adjusted so that each component phospholipid resonance exhibited a small, but measurable, difference in amplitude. This was done to enhance signal visibility so that signals could be followed as they crossed over one another during titrations. The differences in the concentrations of the phospholipids were less than 10%.

The selectivity of some phospholipids, particularly CL, PA and PS, for alkaline earth cations was high enough that only one phospholipid at a time showed a change in chemical shift in the course of a titration. For example, all PA was noted to bind the ions before the tritration of other phospholipids began; thus, small concentration differences between the phospholipids were irrelevant.

Precipitates did not form until samples were very heavily loaded with alkaline earth cations. Titrations were not carried out beyond the point where precipitation began. Other titrations were carried out using samples containing two, three or four phospholipids in equal molar ratios to verify various observaqtions made on the rather complex nine-component phospholipid system. The simpler systems gave results that were qualitatively identical to those observed on the nine-component phospholipid system. However, quantitative differences were observed, particularly between those phospholipids interacting strongly with Mg^{2+} and Ca^{2+} ions.

Alkali ion phospholipid preparations. The chloroform/methanol/aqueous-Cs-EDTA phospholipid mixture used in this study was formulated for the analysis of phospholipids by ^{31}P NMR spectroscopy (9,11). When formulated using the Cs salt of EDTA at pH 6, the second part of the two-part reagent (referred to as Reagent B), consisting of methanol/aqueous 0.2 M Cs-EDTA, is stable at room temperature indefinitely. When formulated using the K salt, the solution is reasonably stable for about 1 h before the K-EDTA salts begin to crystallize from the solution. The corresponding Na-EDTA preparation is unstable, crystallization of Na-EDTA salts proceeds rapidly after mixing the methanol and aqueous salt solutions to generate the methanolic-EDTA scrubbing reagent. Nevertheless, a short period of time (less than 1 min) exists when the Na reagent can be used for quantitative measurements.

Upon mixing Reagent B with Reagent A, the reaction mixture separates into two phases, a major chloroform phase and a minor aqueous phase containing some methanol. The Na salts of EDTA remain soluble in this aqueous phase for at least 10 min before crystallizing from the solution. This permits the sample to be scrubbed thoroughly.

Common stock solutions of pure phospholipids and mixtures of phospholipids in chloroform were used to provide the samples for the alkali metal ion chemical-shift analysis. Prior to preparation of the NMR samples using the NMR scrubbing reagent described above, the samples in chloroform/methanol (2:1, vol/vol) were pre-washed with 0.06 M EDTA solution having the appropriate alkali metal cation at pH 6 and allowed to separate into two liquid phases overnight (9). The chloroform phase was then withdrawn from the separatory funnel, evaporated to dryness

by rotary evaporation at 40°C, and dissolved in the chloroform Reagent A prior to mixing with Reagent B for ^{31}P NMR analysis.

^{31}P NMR spectroscopy. The NMR spectrometer used in this investigation was a multinuclear GE 500 NMB system operating at 202.4 MHz for ^{31}P. Analytical samples were placed in standard, 10-mm (spinning) NMR sample tubes and analyzed using procedures previously published (9). Chemical shifts (δ) are reported in ppm relative to 85% orthophosphoric acid (20,21); however, the primary internal standard was benzene-d_6 used for field-frequency locking purposes. At the beginning of each tritration, the chemical-shift offset value was set to position the PC resonance at −0.84 ppm (9).

RESULTS

A representative ^{31}P NMR spectrum of the nine phospholipid classes examined in this study is shown in Figure 1. The sample is a complex rat heart total lipid extract to which additional PG and PA were added. The principal resonance signals examined in this study are identified (9).

Calcium titrations. Because the interaction of Ca^{2+} ions with phospholipids does not result in noticeable broadening of phospholipid resonance signals, Ca^{2+} ion interactions will be described first. Information derived from spectra in which signals are not completely broadened out permits straight-forward interpretations (12,13, 15,22). Chemical-shift changes to the first titration end point are large with Ca^{2+} relative to the precision of the NMR chemical-shift measurements (202.4 MHz) which gives a high degree of certainty to extrapolations of the linear segments of the resultant chemical-shift titration plots. A typical chemical-shift excursion to the first end point is *ca.* 1 ppm; the precision of the NMR chemical-shift measurement is ±0.001 ppm. The phospholipid solutions also can accept relatively high Ca^{2+} ion levels before precipitation occurs. This allows the titrations to be carried to sufficiently high Ca^{2+} concentrations to firmly establish titration end points.

Titration of phospholipid preparations with aqueous $CaCl_2$ causes the phospholipid signals to be shifted upfield to lower δ values, *i.e.,* in the direction of increased shielding. In this regard, these titrations are similar to pH titrations on phosphates, where the δ value of resonance groups accepting the proton shifts upfield with lower pH levels (16,20,22,23). Phospholipid ^{31}P NMR signals are not quenched by Ca^{2+} ions. Further, noticeable signal broadening does not occur until just prior to precipitate formation. For each phospholipid, a data set for δ as a function of added Ca^{2+} ion can be obtained.

To quantitatively relate the relative chemical-shift change of one phospholipid to that of another, two types of normalization procedures and one correction are required to adjust the data sets. i) Each phospholipid shows a characteristic resonance on the δ scale dependent on the functional group. ii) The magnitude of the upfield shift excursion of each resonance upon Ca^{2+} addition depends on the phospholipid, *i.e.,* the maximum excursion of the chemical-shift per transition is a function of the structure of the complex formed (24-26). Generally, the shift transition to the first end-point is about −1.5 ppm, but may vary among phospholipids over a range of −1.5 ± 0.5 ppm. iii) Initially, the reaction solvent is biphasic. The liquid phases must be allowed to separate to allow removal of the minor aqueous phase before a titration can begin. Otherwise, the residual EDTA present in the aqueous phase will take up the initial Ca^{2+} ions, which would result in a shift of the titration curves towards higher Ca^{2+} levels.

The application of correction and normalization procedures is illustrated in Figure 2. The example is for a pure preparation of PS in the NMR phospholipid reagent (4 μmoles in 3.0 mL). The first step is to normalize the chemical-shift data set so that the curve passes through zero change in the chemical shift ($\Delta\delta$) at the 0 point in the Ca^{2+} ion titration. The intrinsic chemical shift of pure PS in the NMR solvent is −0.050 ppm relative to 85% phosphoric acid (9). To normalize the PS data set, 0.050 ppm is therefore added to each value in the original data set. This normalization is carried out for each phospholipid data set. For PA and CL, which exhibit fundamental δ values of 0.317 and 0.193 ppm, respectively, values of −0.317 and −0.193 are added. This procedure results in a curve similar to the top curve in Figure 2. Such a curve passes through zero on the ordinate, which corresponds to the change in chemical shift, $\Delta\delta$, with no Ca^{2+} ions added. Metal-ion titration curves exhibit two or more linear segments with corresponding transition zones, which are typical of phosphate ^{31}P NMR spectroscopic titrations (12-14,22,27,28).

The residual EDTA in the sample chelates the alkaline earth cations that were added initially (29). Extrapolation of the first linear segment of the top curve to the ordinate gives the amount of curve displacement resulting from

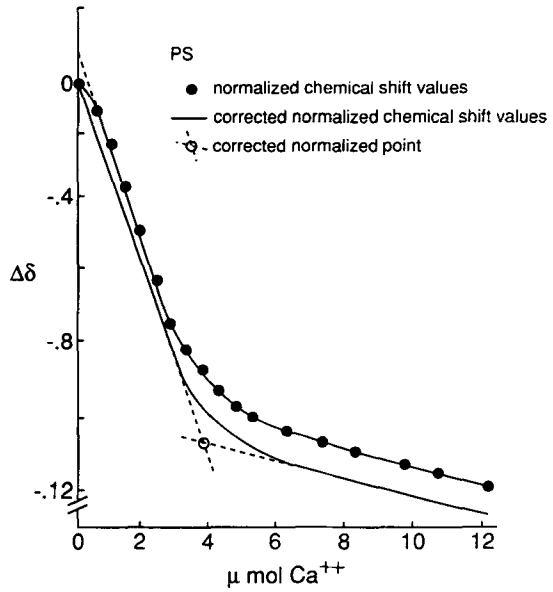

FIG. 2. The ^{31}P NMR chemical shift of PS (4 μmoles/3 mL) as a function of added aqueous $CaCl_2$ (1 M, pH 7.2). The top curve shows the measured chemical-shift difference from the 0-point chemical-shift value (δ 0.193 ppm) of PS. The bottom curve was derived from the top curve after correcting for the error (0.084 ppm) introduced by residual EDTA in the sample. The intersecting extrapolated lines indicate the chemical-shift-difference value and the Ca^{2+} ion concentration corresponding to the first detectable end-point in the titration. Precipitation occurs at Ca^{2+} levels in excess of 25 μmoles.

the residual EDTA in the sample, *e.g.*, 0.084 ppm for PS. The intrinsic-shift-normalized data set is then corrected for this displacement by subtracting 0.084 ppm from each titration-point value. These data correspond to the lower curve in the Figure, which represents the chemical-shift effect of titrating a pure, uncontaminated sample of PS by the indicated quantities of Ca^{2+} ions.

Extrapolation of the linear segments of this corrected curve through the transition zone yields a point corresponding to a hypothetical one-to-one complex of Ca^{2+} and phospholipid (12,13,28). Most important, the total chemical-shift displacement corresponding to formation of this complex (−1.106 ppm in this instance for PS) also is obtained.

Perfect agreement with theory would have the intersection in Figure 2 occur at 4 μmoles added Ca^{2+} ions. Further, at higher than 4 μmole Ca^{2+}, the curve would be horizontal if no further interactions of the phospholipid with Ca^{2+} ions were to take place. The negative slope of the second line segment indicates the presence of additional Ca^{2+}-ion-phospholipid interactions.

The corresponding first-transition chemical-shift displacements for CL and PA are −1.690 and −1.590 ppm, respectively. These are considerably larger values than the −1.106 ppm displacement exhibited by PS (9). To compare the relative interaction potential of these three phospholipids for Ca^{2+} ions, the curves need to be normalized a second time for the displacement corresponding to the initial Ca^{2+}-phospholipid interaction. This is done by dividing each $\Delta\delta$ value of a normalized-corrected data set by the total chemical-shift excursion derived from the extrapolated line segments obtained above. Multiplying these fractional values by 100 yields a scale in percent of total chemical-shift change, as illustrated in Figure 3. For PS, CL and PA these devisors are $\Delta\delta$ −1.106, −1.690 and −1.590 ppm, respectively.

Figure 3 shows the relative effect on chemical shift of titrating a sample containing 3 μmole each of PS and CL with Ca^{2+} ions with the data being displayed as percent of chemical-shift change. The curves have shapes similar to that of the lower curve in Figure 2. In these instances, the extrapolated line segments correspond to 100% of the first transition. The observation that the initial segment of the CL curve is shifted to lower Ca^{2+} ion concentrations relative to that of PS indicates that in this system, composed of two pure phospholipids in an organic solvent, CL has the higher interaction potential for Ca^{2+} by a factor of about 1.5 (8.0 μmoles Ca^{2+} *vs.* 5.4 μmoles Ca^{2+}).

Figure 4 presents similar data for PS, CL and PA as components of the complex phospholipid mixture consisting of total rat heart lipids that have been supplemented with PG and PA. Only the initial linear segments of the curves are given. When using the rat heart lipid mixture (which contains neutral lipids, other non-phosphorus polar lipids, as well as phospholipids), the most avid Ca^{2+} binding phospholipids are PS and PA; CL is much less effective in binding Ca^{2+} ions in the total rat heart lipid system than in the pure lipid system containing PS and CL shown in Figure 3. In this instance, PS and PA have the higher interaction potential for Ca^{2+} relative to CL by a factor of 4.7. The PS and PA plots are similar to those obtained using systems containing only pure phospholipids. Therefore, the binding of Ca^{2+} to PS and PA is only slightly affected by other lipids present.

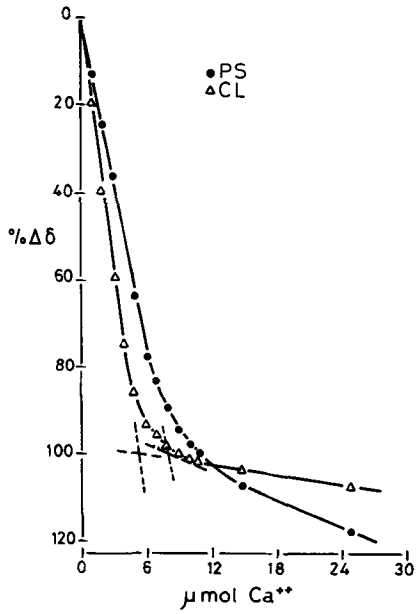

FIG. 3. Percent ^{31}P NMR chemical-shift change of a mixture of PS and CL as a function of added $CaCl_2$. Each phospholipid is 1 mmolar, total solvent volume is 3 mL. The intersecting extrapolated lines at 100% change in chemical-shift indicate the Ca^{2+} ion concentration corresponding to the first end-point in the titration of each phospholipid. In such a plot, the phospholipid with its end-point corresponding to the lowest Ca^{2+} concentration is the more avid Ca^{2+} sequestrant. Precipitation of the sample occurs at Ca^{2+} levels in excess of 25 μmoles.

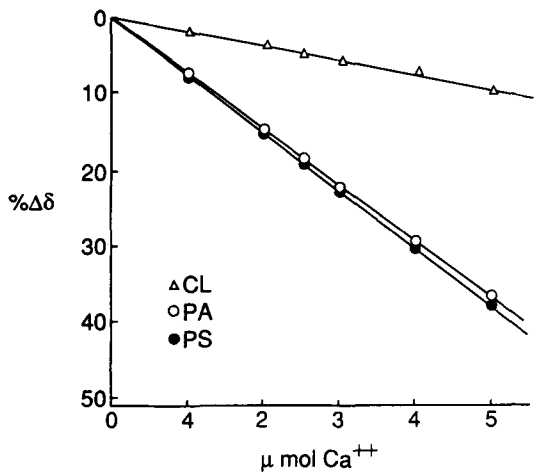

Fig. 4. Percent ^{31}P NMR chemical-shift change of PS, CL and PA, contained in a complex mixture of rat heart lipids, as a function of added $CaCl_2$. Only the initial linear segment of each curve is shown.

However, in the complex rat heart lipid mixture, some other lipid component appears to interfere with the ability of CL to bind Ca^{2+}.

For PG, PI, PE, EPLAS, SPH and PC, end points cannot be determined with reasonable accuracy. The chemical shifts of these phospholipids simply change linearly with added Ca^{2+} ion until precipitation occurs. The titration plots are illustrated, along with the initial linear segments

of the PS, PA and CL curves, in Figure 5. If it is assumed that titration with Ca^{2+} will change all of the phospholipid chemical shifts by about −1.0 ppm, which is a reasonable first approximation, then the relative slopes of the titration line segments presented in Figure 5 give an approximation of the relative interaction potentials of the indicated phospholipids for Ca^{2+} ions. For the complex rat heart lipid system, the phospholipids can be divided into three distinct groups: i) The net charge-neutral zwitterionic phospholipids, PC, SPH, EPLAS and PE, which have a low interaction potential for Ca^{2+}; ii) the anionic phospholipids, PI, PG and CL, which have an intermediate interaction potential; and iii) PS and PA, which have a high interaction potential.

Magnesium titrations. Unlike Ca^{2+}, Mg^{2+} interactions with phospholipids result in a marked broadening of the ^{31}P NMR signal, which can be of sufficient magnitude to quench the phospholipid signal. Therefore, titration curves, such as those presented in Figures 2–5 for Ca^{2+}, usually cannot be obtained for Mg^{2+} with the precision required. However, relative binding interaction potentials of phospholipids with Mg^{2+} ions can be obtained. When the Mg^{2+} ions are added to the sample, the NMR signal of the phospholipid having the highest interaction potential for Mg^{2+} will disappear from the spectral profile first, followed by the signals of other phospholipids according to their binding potential for Mg^{2+} ions until either all the phospholipid signals are quenched or precipitation occurs.

Negative (upfield) chemical-shift changes are observed in the course of Mg^{2+} ion titrations, but these are small compared to those obtained in the Ca^{2+} titrations. For example, in titrating the complex rat heart lipids to the point just prior to precipitation, the following total chemical-shift changes $\Delta\delta$ were observed for those signals that had not been quenched or excessively broadened—PG, −0.032; EPLAS, 0.000; PE, −0.002; SPH, −0.005; PS, −0.078; PI, −0.027; and PC, −0.003 ppm.

Of the nine phospholipids studied, two (CL and PA) showed signal quenching or marked signal broadening upon interaction with Mg^{2+}. The first interaction with Mg^{2+}, which was stoichiometric, became apparent as total quenching of the CL resonance. Subsequently, progressive broadening of the PA signal was observed until precipitation occurred. The remaining phospholipids were essentially unaffected by added Mg^{2+} throughout the observable titration range.

The quenching of the CL resonance by Mg^{2+} is illustrated in Figure 6. The mixture is composed of PA, CL and PS, in the relative amounts indicated by the peak heights shown in the top spectrum, which was obtained on the reagent-scrubbed preparation just prior to Mg^{2+} addition. Total phospholipid content of the sample is approximately 9 μmoles phosphorus. The bottom spectrum, obtained after addition of 3 μmoles Mg^{2+}, shows that the CL resonance has been broadened nearly to the point of extinction (quenched); the PA resonance is slightly broadened, and the PS signal is little affected by added Mg^{2+} ions. Between the two extremes, spectral changes are continuous, at 90 μmoles added $MgCl_2$, precipitation occurs.

During titration of the sample shown in Figure 6, chemical-shift changes occurred that were proportional to

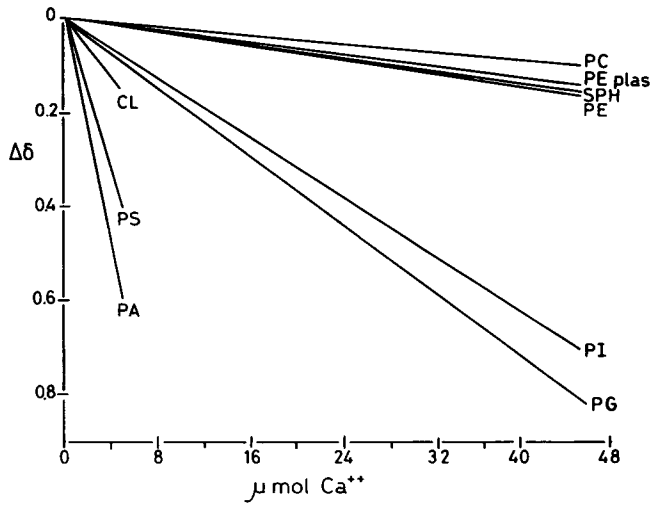

FIG. 5. ^{31}P NMR chemical-shift change of phospholipids in a complex mixture of rat heart lipids as a function of added $CaCl_2$. Only the initial linear portions of the PC, CL and PA curves are plotted. Precipitation occurs at Ca^{2+} levels in excess of 45 μmoles.

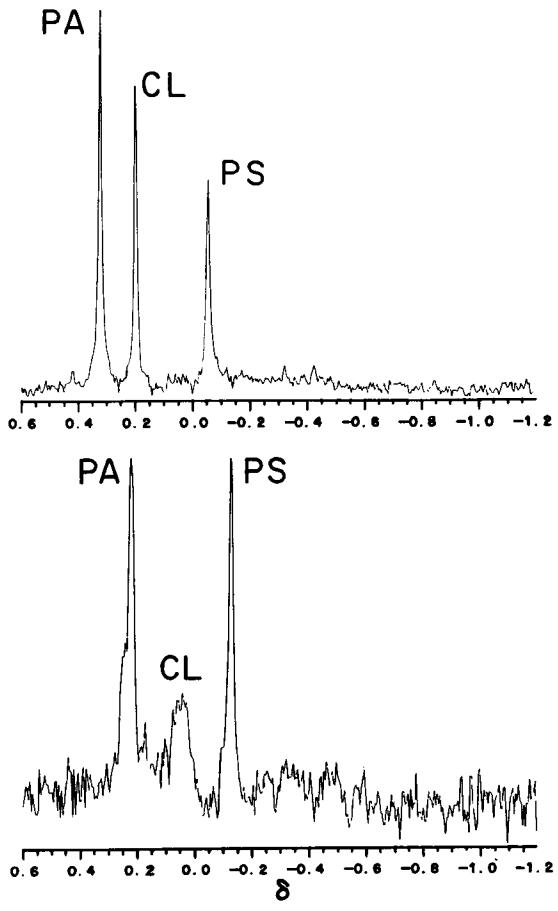

FIG. 6. ^{31}P NMR spectra of a mixture of PA, CL and PS, showing the Mg^{2+} ion quenching of the CL signal and the initial broadening of the PA signal. The top spectrum is that of the phospholipid mixture before addition of $MgCl_2$. The bottom spectrum is that of the same mixture at the point in the Mg^{2+} ion titration just prior to total Mg^{2+} quenching of the CL resonance. Associated with the signal-broadening effects of added Mg^{2+} ions are shifts of the phospholipid resonances to lower δ values.

the amount of added Mg^{2+}. The greatest chemical-shift change was exhibited by CL (−0.15 ppm), followed by PA (−0.10 ppm), followed by PS (−0.07 ppm).

Partition of Mg^{2+} and Ca^{2+} between CL and PS. The above data illustrate that CL shows a preference for Mg^{2+}, while PS shows a preference for Ca^{2+}. In a system containing both CL and PS, as well as Mg^{2+} and Ca^{2+} cations, Mg^{2+} will associate primarily with CL, while Ca^{2+} will associate primarily with PS. This is illustrated in Figure 7.

The top spectrum of Figure 7 was obtained on a sample containing CL and PS. Two well-resolved signals occur. Titration of this sample with an equimolar mixture of Mg and Ca chlorides results in a continuous conversion of the top to the bottom spectrum, with precipitation taking place just after the bottom spectrum was recorded. The CL resonance at this point became substantially broadened, which is a characteristic of Mg^{2+} binding to CL, but not of Ca^{2+} binding to CL. The PS resonance shifted upfield by −0.221 ppm. The direction of the chemical-shift change of PS is characteristic of both Mg^{2+} and Ca^{2+} interactions with PS. The magnitude of the change, however, indicates that Ca^{2+}, and not Mg^{2+}, is the interacting alkaline-earth cation. Using the quenching of the CL signal as the best index of Mg^{2+} binding and knowing the relative concentrations of the reacting components, one can calculate that approximately 90% of the added Mg^{2+} is associated with CL in this binary phospholipid system. On the other hand, it is then reasonable to assume that PS is binding 90% of the added Ca^{2+} ions.

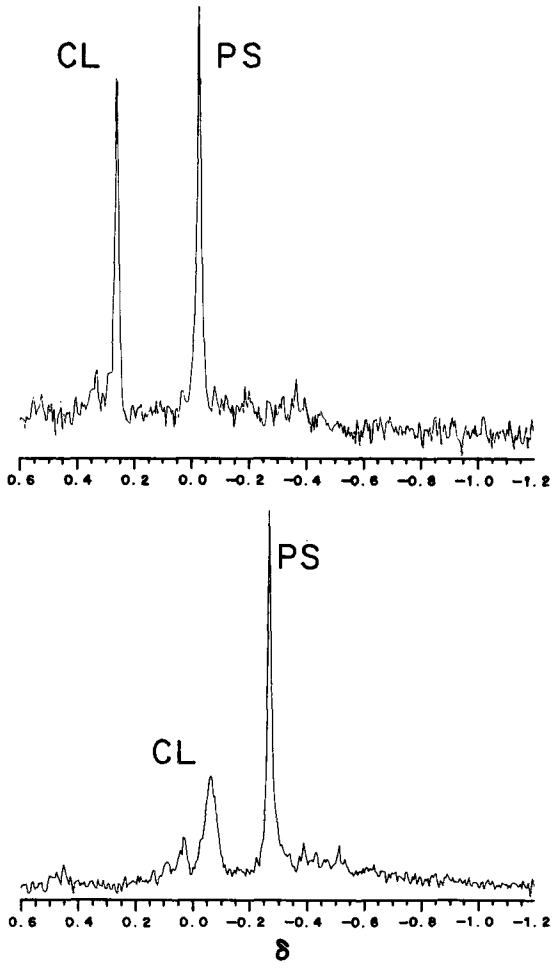

FIG. 7. ^{31}P NMR spectra of a phospholipid sample containing nearly equimolar quantities of CL and PS in the chloroform/methanol NMR reagent. The top spectrum was obtained on the phospholipids prior to the addition of alkaline-earth cations (counter cation, Cs^+). The bottom spectrum was obtained after titrating the sample with an equimolar mixture of the Mg and Ca chlorides to a point just prior to alkaline-earth precipitation of the phospholipids. The CL resonance of the bottom spectrum at δ −0.07 ppm shows the characteristic broadening that results from the interaction of CL with Mg^{2+} ions. Both signals are shifted to lower δ values as a result of the phospholipids interacting with the added alkaline-earth titrating reagent.

Parallel phospholipid interactions with Na^+, K^+ and Cs^+. As the anionic phospholipids are capable of distinguishing between group (II), alkaline-earth, and group (III) (18), cations, with CL preferring to complex with Mg^{2+} and PS preferring to complex with Ca^{2+}, the question arises whether these phospholipids could also distinguish between group (I), alkali ions, such as Na^+, K^+ and Cs^+. Such a distinction would be reminiscent of the selectivity of polyphosphates for alkali metal ions (12,28). The ^{31}P NMR chemical shifts of the anionic phospholipids depend on whether the counter cation is K^+, Cs^+ or $(Me)_4N^+$, but the neutral-zwitterionic phospholipids do not exhibit such an ion-dependence in their ^{31}P NMR chemical shifts (9). As the authors of that study (9) did not report phospholipid chemical shifts with Na^+ as the counter cation, these data have now been added in Table 1.

The data listed in Table 1 were obtained on phospholipids that had been thoroughly washed with Cs^+, K^+ or Na^+ EDTA solutions prepared from the free-acid form of EDTA and 10 M solutions of the corresponding alkali metal hydroxides. The samples also contained the appropriate alkali EDTA Reagent B solution set to pH 7.

Table 1 gives the chemical shifts of the phospholipid in the presence of the alkali metal ions indicated and the absolute differences between the respective chemical shifts. Except for PS, the most negative chemical shifts are obtained for each of the phospholipids with Cs^+ as the alkali ion. Shifts obtained for K^+ are similar to those obtained for Cs^+, with the exceptions that PI, PE, PG and PA resonate at higher chemical-shift values in the presence of K^+. The remaining phospholipids give rise to chemical-shift changes that are small or not large enough to be detected.

When comparing chemical shifts of individual phospholipids in the presence of Cs^+ and Na^+, the anionic phospholipids, PI, CL, PG and PA, yield chemical-shift changes that are greater than δ 0.100 ppm, a finding that also applies to K^+ *vs.* Na^+. PE and EPLAS show a small, positive shift change when either Cs^+ or K^+ are compared to Na^+. The greatest change in comparing Cs^+ or K^+ to Na^+ is exhibited by PA. In order of decreasing magnitude, the chemical-shift change upon switching the counter cation from Cs^+ or K^+ to Na^+ is PA > CL > PI > PG.

Interaction with Na^+ causes a considerable broadening of the PA resonance, from a nominal value of 6 Hz at half-height in the presence of Cs^+ or K^+ to a value >40 Hz in the presence of Na^+. The signal broadening effect

TABLE 1

^{31}P NMR Chemical Shifts and Chemical-Shift Differences of Phospholipids in the Presence of Cs^+, K^+ and Na^+

	^{31}P Chemical shift (pH 7)					
	Chemical shifts (δ)			Difference in δ between cations (ppm)		
Phospholipid[a]	Cs	K	Na	Cs *vs.* K	Cs *vs.* Na	K *vs.* Na
PC	−0.840	−0.840	−0.840	0.000	0.000	0.000
CPLAS	−0.777	−0.777	−0.770	0.000	0.007	0.007
PI	−0.357	−0.281	−0.104	0.076	0.253	0.177
SPH	−0.092	−0.092	−0.090	0.000	0.002	0.002
PS	−0.050	−0.056	−0.052	−0.006	−0.002	0.004
PE	−0.016	0.000	0.015	0.016	0.031	0.015
EPLAS	0.070	0.070	0.080	0.000	0.010	0.010
U	0.130	0.131	0.131	0.001	0.001	0.000
CL	0.181	0.181	0.525	0.000	0.344	0.344
PG	0.527	0.565	0.666	0.038	0.139	0.101
PA	0.283	0.791	1.173	0.508	0.890	0.382

[a]PC, phosphatidylcholine; CPLAS, choline plasmalogen; PI, phosphatidylinositol; SPH, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine; EPLAS, ethanolamine plasmalogen; U, unidentified phospholipid from human lens (17); CL, cardiolipin; PG, phosphatidylglycerol; and PA, phosphatidic acid.

becomes more pronounced with sample dilution, such that at low concentrations the PA signal becomes difficult to detect.

DISCUSSION

One of the many useful features of NMR spectroscopy is that complex mixtures can be directly analyzed (19,21,30). Thus, for example, in examining the spectrum of rat heart phospholipids, all of the common phospholipids can be qualitatively and quantitatively analyzed in a single sample (Fig. 1). This advantage of NMR spectroscopy was exploited in the titration experiments presented herein, because it appeared likely that the lipids would interact (and, for example, aggregate) (9,11) in ways that would alter their chemical and physical properties. Therefore, the focus of this study was not the measurement of phospholipid-alkaline-earth interactions on single phospholipids, but the measurement of phospholipid/Ca^{2+} and phospholipid/Mg^{2+} interactions in complex lipid mixtures.

Interactions with Ca^{2+}. Phospholipids interacting with Ca^{2+}, as measured on the rat heart lipid preparations, fall into three groups (Fig. 5)—PS and PA; CL, PI and PG; and EPLAS, PE, SPH, and PC. Both PS and PA (at pH 7.2) are anionic phospholipids that possess two negative charges per headgroup or per molecule. The anionic phospholipids CL, PI and PG possess one negative charge per phosphate group. Both PI and PG also possess one negative charge per molecule, and the head groups of both are polyols. By contrast, CL carries two negative charges. CL is not a polyol, but contains one free hydroxyl group in the polar head. Of the remaining phospholipids tested, EPLAS, PE, SPH and PC possess zwitterionic polar head groups that, at physiological pH, are electrically neutral. Interactions of Ca^{2+} with this group of neutral phospholipids are minimal. Interactions of PS and PA with Ca^{2+} are the strongest and appear to be medium-independent. The intermediate group is represented by the polyols PI and PG. The phospholipid CL represents a special case because in these mixtures of lipids in organic solvents, CL behaves like a mono-anionic polyol. By itself, or with one or two other phospholipids, CL behaves like a di-anionic phospholipid (Fig. 4). The observed hermaphroditic behavior of CL in organic solvents could be relevant to its biological function.

CL can be viewed in either of two ways: i) As the diphospho, di-anionic molecule; or ii) as the mono-phospho, mono-anionic half-molecule. Viewed as the half-molecule, CL is similar to a polyol with a low interaction potential for Ca^{2+} ions. Viewed as the whole molecule, CL is a dianion with a high interaction potential for Ca^{2+} ions. The key functional group in this coordination complex appears to be the lone and unique β-hydroxyl group on the central glycerol moiety. As diagrammed by Shah and Schulman (2), this hydroxyl group may form a co-ordinate covalent bond with Ca^{2+} in the Ca^{2+}/CL complex. Formation of this bond promotes the participation of the two PO_4 groups, thereby facilitating the observed Ca^{2+}-ion-induced compression of aqueous CL monolayers (2).

Intramolecular hydrogen-bonding of this hydroxyl with one of the CL phosphate groups would have the effect of interdicting formation of the Ca^{2+}/CL complex. It is known that hydrogen bonds form readily with the PO^4 group of phosphate diesters. From crystallographic evidence (20) and molecular mechanics calculations (31), bonding can be either to the equivalent ionic oxygen pair (32) or to the ester oxygen pair; either type being formed with nearly equal probability. In the absence of spatial constraints, both types of bonding may occur in the same complex.

Insertion of a second lipid between the CL fatty acid side-chain pairs would also have the effect of interdicting formation of the Ca^{2+}/CL complex by lowering the probability of both phosphate groups being suitably oriented for Ca^{2+} complex formation. Moreover, complexation would be inhibited further if this interstitial lipid also contained a hydroxyl group available for hydrogen-bonding to the remaining phosphate group of the CL phosphate pair. An excellent candidate for such a lipid is cholesterol. High cholesterol levels of 20% by weight are required for

the expression of the enzymatic activities of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger or the Na^+,K^+-ATPase reconstituted into PC/PS vesicles. The sterol requirement is highly specific for cholesterol (32). Cholesterol, therefore, may act as an inhibitor of Ca^{2+}/CL complex formation in these organic solutions by keeping the CL polar head group in an extended form, or by interacting with one of the CL phosphates through a hydrogen bond.

Interactions with Mg^{2+}. Two of the phospholipids, CL and PA, interact strongly with Mg^{2+} ions, the other phospholipids tested interact weakly or not at all. CL is by far the strongest complexing lipid, with PA not interacting with Mg^{2+} until essentially all of the CL has been Mg^{2+}-saturated. Unlike complexation with the Ca^{2+}, complexation with the Mg^{2+} does not appear to be dependent on the presence of other lipids in the medium. The Mg^{2+} ion complex, therefore, is stronger than the Ca^{2+} ion complex and should form preferentially in the presence of Ca^{2+} ions. It is known that Mg^{2+} has a strong propensity to assume a six coordination, whereas Ca^{2+} preferentially assumes a coordination greater than six. Also, water molecules attached to magnesium ions are held more rigidly than those attached to calcium ions, with Ca^{2+} more readily exchanging water for negative ligands (32).

The above hypothesis was tested in the partitioning experiment presented in Figure 7, where the ^{31}P NMR signal quenching effect of Mg^{2+} was expressed only for the CL signal. The signal of PS exposed to both Mg^{2+} and Ca^{2+} ions only exhibits the alkaline-earth ion upfield shift, but undergoes no signal broadening. The phospholipids CL and PS in combination, therefore, are capable of partitioning Ca^{2+} and Mg^{2+} ions between them in the organic solvent without the participation of other factors. In a study (18) reported on Al^{3+} as the interacting cation, the most avid Al-complexing phospholipid was found to be PA, with CL following PA, and PS following CL.

Interactions with the alkali metal ions Na^+, K^+ and Cs^+. The studies on the ability of phospholipids to discriminate between the alkaline-earth cations in organic solvent were extended (12) to include the alkali metal ions, Na^+, K^+ and Cs^+ (Table 1). The ^{31}P NMR chemical shifts of four phospholipids, PA, CL, PI and PG, responded to the presence of Na^+ ions in the solvating medium by exhibiting more positive chemical-shift values, *i.e.*, the resonance chemical-shift positions became deshielded in the presence of Na^+ relative to their positions in the presence of Cs^+ or K^+ ions. All four phospholipids are net negatively charged anions having no zwitterionic component. Three of these, PI, PG and CL, represent a family with free hydroxyl groups present in the polar head group moiety.

The phospholipid undergoing the greatest chemical-shift change upon switching from Cs^+ to either K^+ or Na^+ is PA. PA being a phosphomonoester, its chemical shift is influenced by two factors: i) Coordination of the phosphate to the cation; and ii) the pK_a of the phosphate group. Alkali metal cation coordination of the phosphate will alter the average bond angles of the phosphate, the magnitude of the average π-bond contribution of the phosphate group to the complex and the magnitude of the average σ-bond contribution, all of which contribute to the ^{31}P NMR chemical shift (24–26). Phosphomonoesters are weak acids having pK_a values in the pH range of 6–8 (23). A change in the pK_a upon coordination of an alkali metal cation (18,33) will alter the ratio of the acid and base forms of the phosphate and, thereby, the average chemical shift of the phosphate functional group. The relative contribution of factors i) and ii) to the phosphate chemical-shift change upon switching counter-cations cannot be rigorously assessed. All that can be said is that the chemical shifts change upon switching from Cs^+ to K^+ and Na^+ ions. Whether the PA phosphate exhibits a preference for one cation over another cannot be determined from the data presented in Table 1 alone.

For the phospholipids PI, PG and CL, the interpretation of shift data is more certain. Because these phospholipids are phosphodiesters, they are strong acids at physiological pH, and a shift contribution by factor ii) does not exist. Moreover, because the chemical shifts of these phosphates change little or not at all upon switching from Cs^+ to K^+ cations, in a first approximation the assumption can be made that factor i) also does not change upon switching between these cations or, at least, that there are internal compensations (unlikely) among the three components of factor i) that cause the chemical-shift values to be the same.

Upon switching from either Cs^+ or K^+ to Na^+, CL, PI and PG undergo a marked and characteristic chemical-shift change which decreases in the order CL > PI > PG. Clearly, among all the phospholipids tested, these three interact with the Na^+ ion in a distinctive manner, and this interaction is not due simply to the existence of a net negative charge. The phospholipid PS has a net negative charge under the conditions of measurement, and PS does not undergo a Na^+-induced chemical-shift change. Thus, in the organic solvent used in this study, the three anionic phospholipids, CL, PI and PG, interact with Na^+ in a characteristic manner that is similar among these three phospholipids and different from Na^+ interactions with all of the other phospholipids.

In a first approximation, two effects can be invoked to explain the relative Na^+-induced chemical-shift changes among CL, PI and PG: i) the number of potential coordinating groups per polar head; and ii) the relative electronegativity of these coordinating groups. Phosphodiester phosphates possesses four potential coordinating ligands at physiological pH, the two equivalent ionic oxygen atoms and the two linking ester oxygens, which are similar, but not equivalent, in phospholipids. The polar head group moiety of phospholipids also contains potential coordinating sites in addition to that of the linking ester oxygen. These are the hydroxyl groups of the glycerol and inositol residues for CL, PI and PG.

Bearing in mind that the chemical shift observed in high-resolution ^{31}P NMR represents the weighted time-averaged sum of the contribution of all possible coordination complexes, an argument based simply on the numbers of available coordination sites has merit. PI and PG each contain similar phosphodiester groups possessing four potential coordinating moieties; however, in their polar head groups, PI has available five hydroxyl groups for coordination to Na^+, whereas PG has only two such groups available. In aggregate, therefore, PI possesses nine potential coordinating groups and PG six such groups, and this difference in the number of available coordinating groups may account for the difference in the (average) chemical shielding of the phosphorus atoms of PI and PG upon interacting with NA^+ ions.

CL contains two phosphodiester groups per molecule (eight potential coordinating groups) but only one hydroxyl group on the central glycerol moiety. The total number of potential coordinating groups, therefore, is nine, just as in PI. The chemical-shift change for the CL phosphates upon interaction with Na^+, however, is nearly twice that for the PI phosphate. In this instance, the difference in shielding may be attributed to the relative electronegativity of the phosphodiester groups over that of the inositol hydroxyl groups.

In the organic solvent used in this study, CL, PI and PG interact in a specific manner with Na^+ cations and may be capable of distinguishing Na^+ ions from among the other cations present in this and other media. For example, the three phospholipids as functional components of the plasma membrane may recognize Na^+ specifically and may be capable of distinguishing (34) Na^+ cations from among other cations present.

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METHOD

The Determination of Tissue Ethanolamine Levels by Reverse-Phase High-Performance Liquid Chromatography

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A rapid and sensitive procedure for the determination of ethanolamine levels in mammalian tissues is reported. Ethanolamine was extracted from the tissue with a chloroform/methanol mixture, followed by phase separation. The aqueous phase was subjected to charcoal chromatography and the eluant was derivatized with phenylisothiocyanate. The amount of phenylthiocarbamyl (PTC) ethanolamine in the tissue extract was determined by reverse-phase high-performance liquid chromatography. Quantitation of PTC ethanolamine was linear between 0.1–10 nmol. The pool sizes of ethanolamine in hamster heart, liver and kidney were found to be 1.07, 0.92 and 1.11 μmol/g wet weight, respectively. The sensitivity of the method would allow the determination of ethanolamine in very small tissue samples.
Lipids 27, 560–563 (1992).

Ethanolamine is the precursor for the *de novo* biosynthesis of phosphatidylethanolamine in eukaryotic cells (1). Recently, the role of ethanolamine in the glycosyl phosphatidylinositol membrane anchors has been demonstrated (2). Although ethanolamine can be synthesized *in vivo via* decarboxylation of phosphatidylserine (3), the majority of cellular ethanolamine appears to originate from extracellular sources. The active uptake of ethanolamine by the liver (4), heart (5,6), brain (6), kidney (6), and by cell cultures (7) has been documented. Changes in extracellular ethanolamine have been shown to cause alterations in the intracellular concentrations of ethanolamine which, in turn, may play a role in the direct regulation of phosphatidylethanolamine biosynthesis (7). Changes in ethanolamine levels also may affect the choline uptake and phosphatidylcholine biosynthesis in the heart (8) and retinal tissues (9).

In earlier studies, the level of ethanolamine in a tissue extract was determined using an automatic amino acid analyzer. Briefly, a protein-free tissue extract was obtained and ethanolamine was separated from other metabolites by ion-exchange chromatography. The amount of ethanolamine eluted from the column was quantitated spectrophotometrically after ninhydrin reaction. The method was initially developed for the analysis of ethanolamine levels in serum (10) and later adapted for the determination of ethanolamine levels in tissues (5,11). The major limitation of this method was the difficulty in completely separating ethanolamine from other cellular metabolites. In addition, the sensitivity of the assay was not high enough to allow the determination of ethanolamine levels in very small tissue samples (5). To circumvent these difficulties, several other methods for ethanolamine determination were reported (12–17). However, these methods were typically designed for the quantitation of ethanolamine under very specific conditions and could not be easily adapted for the general determination of ethanolamine in mammalian tissues. In the present study, we report a rapid and sensitive procedure for the determination of intracellular ethanolamine by reverse-phase high-performance liquid chromatography (HPLC).

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Abbreviations: HPLC, high-performance liquid chromatography; PTC, phenylthiocarbamyl; PITC, phenylisothiocyanate.

MATERIALS AND METHODS

Materials. [1-^{3}H]Ethanolamine hydrochloride (8.8 Ci/mmol) was purchased from Amersham International Ltd. (Oakville, Ontario, Canada). Phenylisothiocyanate (PITC) and standard amino acid mixtures were obtained from Pierce Chemical Company (Rockford, IL). Pyridine and triethylamine were from Fisher Scientific (Ottawa, Ontario, Canada). Acetonitrile (HPLC-grade) and other chemicals were purchased from the Canlab division of the Baxter Diagnostics Corporation (Mississauga, Ontario, Canada). Activated charcoal and ethanolamine were from Sigma Chemical Company (Toronto, Ontario, Canada). Celite was a product of Supelco Inc. (Oakville, Ontario, Canada).

Preparation of tissue sample. Syrian golden hamsters weighing 120 ± 20 g were used, and sacrificed by decapitation. Tissue samples of 0.25 g were removed and homogenized in 10 mL of chloroform/methanol (1:1, vol/vol). In some experiments, 1 μCi of labelled ethanolamine was added to the homogenate. The homogenate was allowed to sit at room temperature for 30 min and was then centrifuged at 1,000 × *g* for 10 min. The supernatant was decanted and the pellet was extracted twice with 5 mL of chloroform/methanol (2:1, vol/vol) each time. The extracts were pooled, and the ratio of chloroform/methanol/0.9% KCl in the extract was adjusted to 4:2:3 (vol/vol/vol). The mixture was centrifuged at 250 × *g* to facilitate phase separation. An aliquot (5 mL) of the aqueous phase was evaporated to dryness under a stream of nitrogen, and the sample was dissolved in 0.5 mL of 10 mM sodium phosphate buffer, pH 7.4. The sample solution was applied to a 2 × 0.5 cm charcoal/celite (1:2, w/w) column equilibrated with 10 mM sodium phosphate solution (pH 7.4) containing 2% ethanol. Ethanolamine was eluted from the column with 10 mL of the same buffer. The volume of the eluant was reduced by evaporation.

Coupling of ethanolamine with PITC. Coupling was accomplished by a modified method of Heinrikson and Meredith (18). The buffer in the sample was removed *in vacuo* with a Savant (Farmingdale, NY) SC100 vacuum concentrator, and 200 μL coupling buffer containing acetonitrile/pyridine/triethylamine/water (10:5:2:3, by vol) was added. The solvent in the mixture was removed *in vacuo* and the sample was redissolved in another 200 μL of coupling buffer. The coupling reaction was started by the addition of 5–20 μL PITC and the reaction mixture was allowed to sit at room temperature for 15 min. The

solvent in the reaction mixture was evaporated *in vacuo* and the resulting products were dissolved in 250 μL of water/acetonitrile (7:2, vol/vol).

Analysis of phenylthiocarbamyl ethanolamine by HPLC. A Pharmacia LKB (Uppsala, Sweden) SuperPac Spherisorb 3 μm ODS2 column (4 × 125 mm) equipped with a 3 μm ODS guard cartridge was used in this study. The column was equilibrated with 12.5 mM potassium phosphate, pH 6.4, at a constant flow rate of 1.0 mL/min. After sample application (20 μL), the column was washed for 5 min with the equilibration buffer, followed by a 30-min wash with a linear gradient of 0–40% acetonitrile. After completion of the run, the column was washed with acetonitrile for 10 min and re-equilibrated with the equilibration buffer. The absorbance of the eluant was monitored at 254 nm with an Isco (Lincoln, NE) UV detector equipped with an HPLC cell. Data were collected in the first 30 min of the run, and the area of each peak was analyzed using a Beckman (Mississauga, Ontario, Canada) 450 Data System.

RESULTS AND DISCUSSION

The conversion of ethanolamine into its phenylthiocarbamyl (PTC) derivative was investigated. Known amounts (0.1–2.5 μmol) of ethanolamine were used to react with PITC to form PTC ethanolamine. After the reaction, the unreacted PITC was removed by evaporation *in vacuo* and the amount of PTC ethanolamine formed was determined spectrophotometrically. Based on the molar extinction coefficient of PTC amino acids at 254 nm (18), over 98% of the ethanolamine in the reaction mixture was converted into PTC ethanolamine. The ability to separate and identify PTC ethanolamine by reverse-phase HPLC was examined. Under the conditions outlined in Materials and Methods, PTC ethanolamine was eluted as a single peak with a retention time of 17.5 min (Fig. 1A). When a PTC amino acid mixture (derived from a standard amino acid mixture) was applied to the column, none of the amino acid derivatives displayed a retention time between 16.5–18.5 min (Fig. 1B). When a mixture of PTC ethanolamine and PTC amino acids was applied to the column, PTC ethanolamine was eluted as a single peak with a retention time of 17.5 min (Fig. 1C). The amount of PTC ethanolamine applied *vs.* peak area obtained by reverse-phase HPLC was examined. The quantitation of PTC ethanolamine by peak area was found to be linear when 0.10–10.0 nmol PTC ethanolamine was applied to the column (Fig. 2). The sensitivity of the determination could be further enhanced by an increase in the sensitivity of the detector unit.

A chloroform/methanol mixture was used for the extraction of ethanolamine from the tissue. The tissue was homogenized in 40 vol of chloroform/methanol (1:1, vol/vol) and the homogenate was centrifuged to obtain a clear extract. The pellet was re-extracted twice with chloroform/methanol (2:1, vol/vol). A 0.9% KCl solution was added to the pooled extract to cause phase separation. When labelled ethanolamine was added to the tissue homogenate, 99% of the radioactivity was recovered in the aqueous phase. The partition of ethanolamine into the aqueous phase eliminated the contamination of lipid material in the sample. However, the aqueous phase contained nucleotides and other metabolites whose absorbance at UV range could interfere with the analysis of PTC ethanolamine. The majority of these metabolites were removed by charcoal chromatography. The efficiency of the column to remove these contaminants (99.5%) was monitored by determining the absorbance of the sample at 260 nm and 280 nm before and after charcoal chromatography. The yield of ethanolamine was 93% (Table 1).

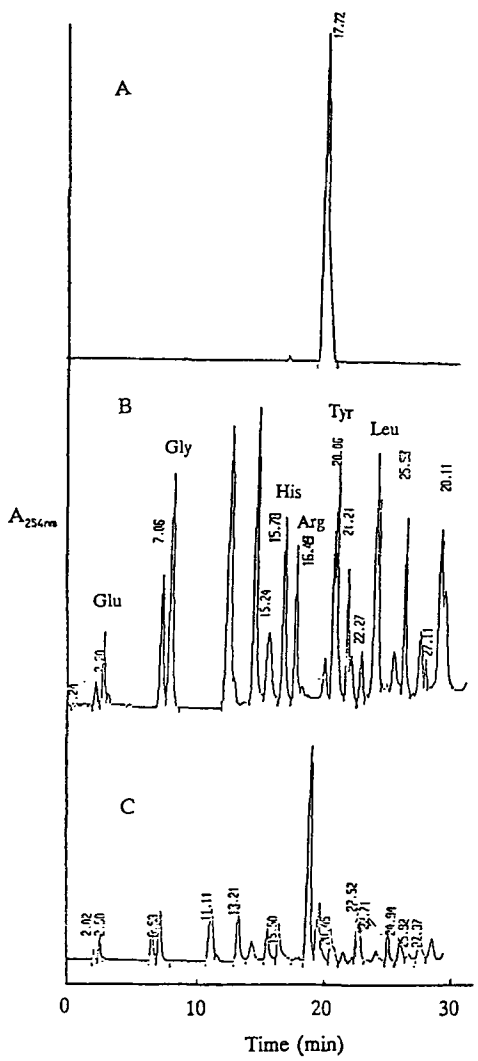

FIG. 1. Separation of PTC ethanolamine by reverse-phase HPLC. Subsequent to sample application (20 μL), the column was washed for 5 min with 12.5 mM potassium phosphate buffer, pH 6.4, followed by 30 min wash with a linear gradient of 0–40% acetonitrile. The flow rate was 1 mL per min, and the numbers shown are the retention times for each peak. A, PTC ethanolamine; B, PTC amino acids; and C, mixture of PTC ethanolamine and PTC amino acids.

For measuring ethanolamine content, two aliquots were taken from the aqueous phase of each sample. A known amount of ethanolamine (100 nmol), which served as an internal standard, was added to one of the aliquots. Both aliquots were reacted with PITC to obtain the PTC derivatives. After reaction, the preparation was resuspended in 250 μL of water/acetonitrile (7:2, vol/vol), and a 20 μL fraction was analyzed by reverse-phase HPLC. A typical chromatogram is shown in Figure 3A. The chromatogram of an identical sample containing the internal standard is depicted in Figure 3B. The internal standard resulted in an increase in the size of the peak correspond-

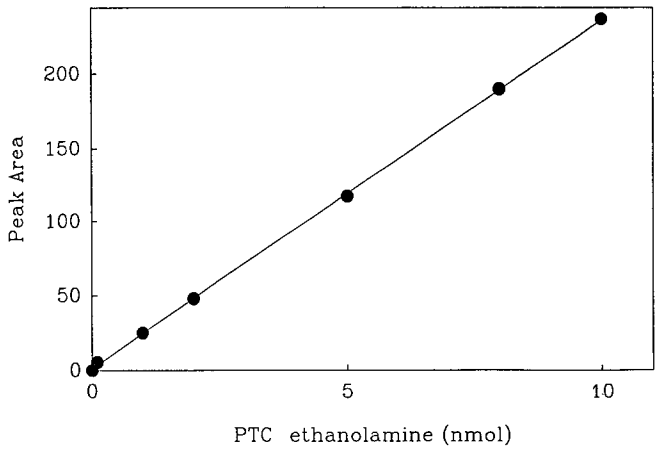

FIG. 2. The quantitation of standard PTC ethanolamine by reverse-phase HPLC. Known amounts of PTC ethanolamine were applied to the column and the corresponding peak areas detected at 254 nm are depicted. Each point is the mean of two separate determinations.

TABLE 1

Ethanolamine Recovery During Pool Size Analysis[a]

Procedure	% Yield[b]
Tissue homogenate	100
Aqueous phase	99
Charcoal chromatography	93

[a][1-^{14}C]Ethanolamine (1 μCi) was added to the tissue homogenate, and the yield after each step was calculated from the amount of radioactivity recovered.
[b]The values are the mean of three separate experiments.

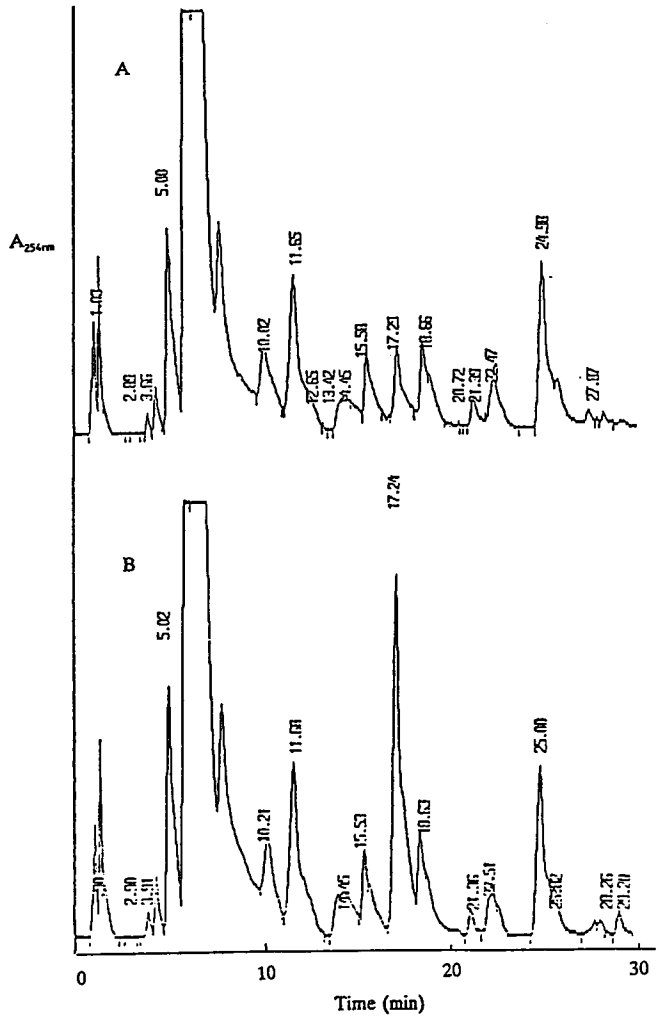

FIG. 3. The quantitation of ethanolamine in hamster heart. Ethanolamine was extracted from the tissue and derivatized to PTC ethanolamine. The derivatized tissue extract was analyzed by reverse-phase HPLC. A, The elution profile of the tissue extract; and B, the elution profile of the tissue extract containing an internal standard of ethanolamine.

ding to the authentic PTC ethanolamine standard with retention time of 17.5 min. The increased peak area of the sample containing the internal standard was 8 nmol (Fig. 2), which corresponds to the amount of internal standard we added to the sample. Our result indicates that all the ethanolamine in the sample was being derivatized to PTC ethanolamine. In some samples, labelled ethanolamine was added to the tissue homogenate and the sample was processed and derivatized as mentioned in Materials and Methods. The eluant from the HPLC was collected into 0.5-mL fractions, and the radioactivity in each fraction was determined. The fraction corresponding to the 17.5 min time point contained the radioactivity which further confirms this peak as PTC ethanolamine.

The pool sizes of ethanolamine in hamster heart, liver and kidney are shown in Table 2. There was no significant difference in the ethanolamine pools between the major hamster tissues. The value obtained for hamster liver was substantially higher than the previously reported value (0.3 μmol/g) for cat liver (11), but the value for the hamster heart was significantly lower than that (5.2 μmol/g) reported earlier (5). The difference in liver ethanolamine could be explained by variation between species. The difference in ethanolamine level in the heart might arise from the methods of quantitation. In the earlier study (5), the amount of ethanolamine was determined by the calibration factor of an amino acid, whereas internal and external standards of ethanolamine were used for calibration in the present study.

TABLE 2

Pool Sizes of Ethanolamine in Hamster Tissues[a]

Tissue	Pool size of ethanolamine
Heart	1.07 ± 0.07
Liver	0.92 ± 0.15
Kidney	1.11 ± 0.32

[a]Each value represents the mean ± SD of three separate sets of experiments, each determined in duplicate. The pool sizes of ethanolamine in hamster tissues are expressed as μmol/g wet weight.

Recently, the determination of ethanolamine in bovine aortic endothelial cells (7) and fetal bovine serum (7) by HPLC equipped with a C_{18} column was reported. Ethanolamine was converted to 2,4-dinitrobenzylethanolamine, and the product was quantitated by reverse-phase

HPLC. However, the sensitivity and specificity of this assay was not defined to allow comparison with the present study. The determination of ethanolamine by PITC derivatization and subsequent reverse-phase HPLC is a facile and rapid procedure to examine the pool sizes of ethanolamine in mammalian tissues. The sensitivity of this procedure is high enough for the determination of ethanolamine in biopsy samples (10–50 mg).

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COMMUNICATION

Production of Eicosapentaenoic Acid by Freshwater *Vibrio*

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Arctic charr, *Salvelinus alpinus* (L.), were fed a moist pellet diet based on casein, dextrin and coconut oil in fresh water. The diet contained substantial amounts of saturated fatty acids, especially 12:0. Polyunsaturated fatty acids in the n-3 series were not detected in the diet, but analysis of the intestinal liquor stripped from the charr showed that eicosapentaenoic acid (EPA, 20:5n-3) accounted for 3.5% of fatty acids in total lipid. Colony forming units (CFU) of aerobic microorganisms were approximately 10^4 per mL intestinal liquor. Seventeen bacterial strains isolated from the intestinal liquor were screened for EPA production. Four isolates contained a high proportion of EPA in their total lipid. These isolates belong to the species *Vibrio*.
***Lipids* 27, 564-566 (1992).**

Bacteria are generally considered not to contain polyunsaturated fatty acids (PUFA); instead such fatty acids are only believed to be formed *de novo* by photosynthetic organisms (1). However, the presence of linoleic acid (18:2n-6) in cellular lipids has been demonstrated in *Vibrio cholera* (2), several *Vibrio* species (3) and in three bacterial species of the human pathogen *Yersinia* (4,5). Eicosapentaenoic acid (20:5n-3) has been reported as a membrane constituent in several marine bacterial species: deep-sea bacteria (6), *Alteromonas* sp. (7) *Shewanella Alteromonas putrefaciens* (8,9) and *Flexibacter* (10). These observations may indicate that microorganisms contain PUFA more frequently than earlier suggested.

No investigations to date have reported that freshwater bacteria contain PUFA. The focus of our study was therefore to investigate whether bacteria isolated from freshwater fish contain PUFA or not.

MATERIALS AND METHODS

Fish. Alevins of Arctic charr, *Salvelinus alpinus* (L.), were reared on commercial feed (Tess Elite Pluss, Skretting LTD, Stavanger, Norway) from the initial feeding stage to an average weight of approximately 10 g. Thereafter the fish were fed the artificial diet.

Diet. The diet consisted of a moist pellet based on casein, dextrin and coconut oil. The composition of the diet is given in Table 1. A detailed description of the different premixes and vitamins added to the diet is given by Olsen *et al.* (11). The diet components were thoroughly mixed, pelleted, and stored at −80°C prior to use.

TABLE 1

Ingredients (g/kg dry weight) of the Diet[a]

Ingredients	
Casein	706.5
Dextrin	170.0
Gelatin	17.0
Premix I	30.0
II	1.7
III	1.8
IV	20.0
Coconut oil	53.0

[a]A detailed description of the added premixes and vitamins is given by Olsen *et al.* (11).

Experimental conditions. We examined 30 Arctic charr in our study, following the experimental design of Ringø and Nilsen (12). This study was undertaken under a natural photoperiod at 70°N from October to April with an average water temperature of 6.0°C ± 1.0°C.

Sampling of intestinal liquor. Ten fish, each weighing about 40 g, were stripped after having been anesthetized in 0.3% benzocaine. The belly of the fish was pressed and intestinal liquor was extruded through the rectum. The intestinal liquor was separated from the fecal pellet after standing for 2 min.

Lipid analyses of diet and intestinal liquor. Analyses of diet and intestinal liquor were carried out in triplicate. Lipids were extracted by the method of Bligh and Dyer (13). Methyl esters of fatty acids were prepared by H_2SO_4 catalyzed transesterification of total lipid in methanol (14), and were analyzed by gas chromatography (Hewlett-Packard, Model 5890 A, Waldbronn 2, Germany) using a SP 2330 capillary column (30 × 0.25 mm i.d.) and helium as carrier gas. The temperature program employed was 60°C for 1 min, followed by an increase of 30°C/min to 180°C for 7 min and thereafter 5°C/min to 240°C. Individual fatty acids were identified by comparison with known standards (Supelco 4-7019, 4-7042, 4-7033, 4-5589, Supelco, Bellefonte, PA) and quantitated using a Hewlett-Packard 3393A integrator. Eicosapentaenoic acid (20:5n-3) was identified by gas chromatography-mass selective detector (GC-MSD) using a 5970 MSD (Hewlett-Packard) connected to a Hewlett-Packard work station 9000-300, and further confirmed by gas chromatography-mass spectrometry (GC-MS) using conditions described elsewhere (15).

Isolation of microorganisms. Total viable counts were performed on TSAg plates containing TSA (tryptic soy agar) 40 g/L and 5 g/L glucose. Intestinal liquor was diluted in sterile 0.9% saline; 0.1 mL vol of appropriate dilutions were spread on the surface of the TSAg plates. The plates were incubated at 12°C, and inspected daily for up to 4 wk. After enumeration, a representative selection of colonies was subcultured on TSAg plates. After

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Abbreviations: CFU, colony forming units; EPA, eicosapentaenoic acid; GC, chromatography; MSD, mass selective detector; PUFA, polyunsaturated fatty acids; TSA, tryptic soy agar; TSB, tryptic soy broth.

confirmation of culture purity, bacteria isolates were identified to species level by standard biochemical tests (16).

Lipid analyses of microorganisms. Seventeen bacterial isolates, isolated from intestinal liquor at 12°C were grown in lipid-free TSBg medium at 12°C to an optical density (OD_{600}) of approximately 0.7. Thereafter, sterile culture medium (TSBg) was inoculated with 0.5% (vol/vol) of each bacterial isolate and the cultures were maintained at 12°C. Microorganisms were harvested in stationary growth phase. The bacteria were centrifugated at 5.000 rpm for 15 min in a Sorvall centrifuge (model RC2-B), and then suspended in 5 mL methanol. The bacterial cells were sonicated for 1 min and extracted with chloroform/methanol (2:1, vol/vol). The extract was left at 12°C for several hours, and then the solvent was evaporated (17). Methyl esters were prepared as described above.

RESULTS AND DISCUSSION

Fatty acid compositions of the lipids from diet and intestinal liquor are presented in Table 2. The diet had a high content of saturated fatty acids which accounted for approximately 90% of total fatty acids. The major saturated fatty acid was 12:0 (about 41% of total fatty acids). Smaller proportions of the monoene fatty acids (16:1n-7 and 18:1n-9) were also detected. Polyunsaturated fatty acids were not detected in the dietary lipids. Eicosapentaenoic acid (EPA; 20:5n-3) was, however, detected in intestinal liquor, at a level of 3.5% of total fatty acids (Table 2). In addition, high proportions of 20:1 (7%) and 22:1 (9%) were detected. Colony forming units (CFU) of aerobic microorganisms in intestinal liquor were approximately 10^4 when the intestinal liquor was incubated on TSAg plates at 12°C. The EPA productivity of 17 bacterial isolates, isolated from intestinal liquor was tested. Four bacterial isolates tested produced EPA when grown aerobically in a lipid-free TSBg medium. These bacterial isolates belong to *Vibrio* spp. The highest production of cellular EPA, approximately 10% of total fatty acids, was detected in *Vibrio* isolate B10 (Table 3). The relative high quantity of EPA is of bacterial origin, because neither EPA nor other polyunsaturated fatty acids (PUFA) were detected in the growth medium (results not shown). Moreover, fatty acid composition of a *Vibrio* spp. isolated from the intestinal liquor not producing EPA is presented in Table 3.

The proportion of EPA detected in cellular lipid of *Vibrio* isolate B10 in the present study was considerably lower than the values reported for several deep-sea bacteria (25–37%) (6) and for marine bacteria *Shewanella Alteromonas putrefaciens* (24%) (8).

Erwin and Bloch (18) demonstrated that cyanobacteria contained 18:3 PUFA, but C_{20} and C_{22} PUFA were not found in this group. Based upon the observation of Erwin and Bloch (18), and their own observation that C_{20} and C_{22} PUFA were present in deep-sea bacteria (6), DeLong and Yayanos (6) put forth a hypothesis that the genes for PUFA biosynthesis evolved separately in deep-sea bacteria. However, since the freshwater *Vibrio* examined in the present study contained a relatively high proportion of C_{20} long-chain PUFA, the ability to synthesize PUFA may be more universal than generally believed.

TABLE 2

Fatty Acid Composition (%) of the Diet and Intestinal Liquor[a]

	Diet[b]	Intestinal liquor
Total saturated acids	88.2	41.0
8:0	2.1	1.7
10:0	4.8	4.1
12:0	40.8	7.7
14:0	17.3	1.6
15:0	nd	0.5
16:0	12.1	18.8
18:0	11.1	6.6
Total monoenoic acids	6.3	42.8
16:1n-7	0.4	1.5
17:1	nd	4.3
18:1n-7	5.9	18.6
20:1	nd	7.0
22:1	nd	9.0
24:1	nd	2.4
20:5n-3[c]	nd	3.5

[a]Values are means of three samples.
[b]nd, not detectable.
[c]Identified by GC-MS.

TABLE 3

Fatty Acid Composition of Total Lipid from Five *Vibrio* spp. Isolated from Intestinal Liquor[a,b]

	Isolate number				
	A2	A7	B7	B10	B17
13:0	0.6	0.9	0.8	1.0	0.8
14:0	1.6	2.4	1.3	0.9	1.0
15:0	trace	2.9	1.7	1.6	3.1
16:0	27.6	9.4	8.6	11.1	9.2
17:0	trace	0.4	1.7	2.1	2.1
18:0	trace	trace	0.9	0.7	0.5
a-13:0	0.8	0.5	trace	0.6	trace
i-15:0	trace	9.7	12.3	9.1	11.7
a-15:0	nd	0.5	trace	1.5	0.5
i-17:0	nd	trace	0.7	trace	1.5
15:1n-5	nd	1.4	0.6	0.9	0.8
16:1n-9	4.0	trace	3.9	2.1	1.4
16:1n-7	52.4	43.4	26.4	26.4	28.1
17:1n-10	nd	trace	0.5	0.9	trace
17:1n-8	nd	5.3	8.9	7.2	10.0
17:1n-6	nd	1.3	2.0	2.3	1.9
18:1n-9	0.5	1.3	6.3	6.8	7.8
18:1n-7	10.7	4.0	5.4	8.3	4.5
19:1n-8	nd	trace	trace	0.8	0.7
20:5n-3[c]	nd	10.7	7.9	7.9	6.3

[a]nd, not detectable. [b]Trace (<0.5%). [c]Identified by GC-MS.

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Catalytic Deuteration of Alkynols and Their Tetrahydropyranyl Ethers[1]

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To ascertain the effect of a replaceable hydrogen on deuterium incorporation, 2-pentynol, 2-hexynol, 3-hexynol, their tetrahydropyranyl (THP) ethers, and the mono- and di-THP ethers of 3-hexyne-1,6-diol were deuterated catalytically with deuterium gas. The differences in percent d_4 (using Wilkinson's catalyst, *tris*(triphenylphosphine)-chlororhodium) or percent d_2 (using Lindlar's catalyst, Pd on $CaCO_3$ poisoned with a lead salt) measured for the alcohols and their THP ethers were, in general, less than 4% with either catalyst. Noticeable exceptions occurred during the deuteration over Wilkinson's catalyst of 2-pentynol and 2-hexynol; extensive scatter resulted in a substantial decrease in percent d_4. This scatter was not observed with the corresponding THP ethers.
Lipids 27, 567–569 (1992).

Over the years we have synthesized (1–4) a number of fatty acids labelled with deuterium. The deuterium was introduced catalytically either with Wilkinson's catalyst, *tris*(triphenylphosphine)chlororhodium, or Lindlar's catalyst, Pd on $CaCO_3$ poisoned with a Pb salt. These catalysts have proven useful for the preparation of multigram quantities of fatty acids with high isotopic purities. From early observations we concluded that substantially greater deuterium incorporation and less scatter could be obtained by elimination of replaceable hydrogens on the molecule prior to the catalytic deuteration. Elimination of the hydroxy hydrogen was accomplished by converting alkynols to their tetrahydropyranyl (THP) ethers. The objective of this study was to measure the increase in percent d_2 or percent d_4 that would be realized by deuterating the THP ether rather than the alcohol.

EXPERIMENTAL PROCEDURES

Materials. *Tris*(triphenylphosphine)chlororhodium was obtained from Strem Chemical Company (Newburyport, MA). Lindlar catalyst and quinoline were purchased from Aldrich Chemical Company (Milwaukee, WI). 3-Hexynol, 2-hexynol and 2-pentynol were obtained from Farchan Laboratories (Gainesville, FL). Deuterium (99.5%) was purchased from Matheson (Joliet, IL). The mono-THP ether of 3-hexyne-1,6-diol was prepared according to Raphael and Roxburgh (5).

Mass spectral analyses. The deuterated pentynol and hexynols, and their THP ethers were analyzed by gas chromatography-mass spectrometry using a Hewlett-Packard (Avondale, PA) model HP 5890 Series II Gas Chromatograph and a Hewlett-Packard model HP 5988A mass spectrometer. The samples were ionized by the chemical ionization technique using isobutane as the reagent gas. The data were recorded using selected ion monitoring (SIM) of the most prominent peaks. The masses chosen for SIM included all mass numbers in the prominent peak clusters of both the non-deuterated standards and the deuterated compounds. A sample of the nondeuterated standard compound was run prior to the deuterated compound. The deuterium distribution was calculated by solving the simultaneous equations set using the unknown deuterium distribution values, the pattern of intensities of the standard and the measured mass intensities (6). Since there was some separation of the deuterated components, with the compound with the highest number of deuterium atoms eluting first, the gas chromatograms were cut very broadly, down to the baseline.

Deuteration with Wilkinson's catalyst. Benzene (50 mL) was placed in a 100 mL round-bottom flask equipped with a magnetic stirrer and attached to an apparatus designed for maintaining one atmosphere of pressure and for measuring the amount of gas absorbed. The system was evacuated and flushed with nitrogen (3X) and with deuterium (2X). Stirring was stopped, *tris*(triphenylphosphine)chlororhodium (0.2 g) was added and the system was evacuated and flushed twice with deuterium. Stirring was started and as the catalyst was reduced, the solution developed the color of iced tea. The stirrer was stopped, the substrate (1 g) was added and the system was evacuated and flushed twice with deuterium. Stirring was resumed and continued until absorption of deuterium ceased (1.5–3 h). Benzene was removed on the rotary evaporator, the residue was diluted with hexane (30 mL) and the reddish precipitate was separated by filtration and by passage through a SEP PAK silica cartridge. Removal of the solvent left the product.

Deuteration with Lindlar's catalyst. The substrate (1 g) was dissolved in hexane (50 mL) in a 100 mL round-bottom flask equipped with a magnetic stirrer and attached to an apparatus designed for maintaining one atmosphere of pressure and for measuring the amount of gas absorbed. Lindlar's catalyst (100 mg) and quinoline (50 μL) were added and the system was evacuated and flushed twice with deuterium. Stirring was continued until deuterium absorption ceased (30–60 min). The reaction mixture was filtered through a pad of Celite, washed with 5 mL cold 5% HCl, 5 mL saturated $NaHCO_3$, and 2×5 mL H_2O, and dried over anhydrous sodium sulfate. Removal of the solvent left the product.

The reaction of 2-pentynol and 2-hexynol with deuterium over Lindlar catalyst was very slow, and repeated additions of catalyst were required for it to proceed.

RESULTS AND DISCUSSION

Catalytic hydrogenation of alkynes and alkenes over heterogeneous (insoluble) catalysts usually results in isomerization (7,8). Several homogeneous (soluble) catalysts

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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Abbreviations: SIM, selected ion monitoring; THP, tetrahydropyranyl.

have been reported to not give isomerization. One of the most common is Wilkinson's catalyst, *tris*(triphenylphosphine)chlororhodium (9–11). The preferred solvent for this catalyst is benzene because it has been noted (12,13) that positional and geometric isomerizations do not occur in this solvent but do occur in alcohol or benzene alcohol solutions (13,14). Table 1 lists the deuterium contents obtained from the catalytic deuteration with Wilkinson's catalyst of several alkynols and their THP ethers. Deuteration of 3-hexynol and its THP ether (1-1 and 1-2) gave high d_4 values; the difference in d_4 incorporation was about 4.5%. Deuteration of the mono- and di-THP ethers of 3-hexyne-1,6-diol (1-3 and 1-4) likewise gave high d_4 values; the difference in this case was less than 2%. For these compounds there is little to be gained by preparing and reducing the THP ether rather than by reducing the alkynol directly. With 2-hexynol and 2-pentynol and their THP ethers (1-5 through 1-8) the situation is quite different. With the THP ethers high d_4 incorporation was obtained; better than 90% in both cases. With the alcohols, 2-pentynol and 2-hexynol, however, there was extensive scatter and low values of d_4. Meese and Borstel (15) reported extensive scattering due to allenic rearrangements during heterogeneous catalytic deuteration of the THP ether of 2-butynol in ethyl acetate. Homogeneous catalytic deuteration of this THP ether with Wilkinson's catalyst in ethyl acetate did not produce any scrambling. They did not investigate the deuteration of 2-butynol with this catalyst. To prepare pentanol-2,2,3,3-d_4 or hexanol-2,2,3,3-d_4, the route through the THP ether is obviously to be preferred.

Lindlar's catalyst is a popular catalyst for semihydrogenation of the triple bond (16). Table 2 lists the deuterium contents obtained from catalytic deuteration with Lindlar's catalyst of several alkynols and their THP ethers. Deuteration with Lindlar's catalyst of 2-pentynol was carried out in three different solvents, hexane, diethyl ether, and benzene (2-1, 2-2, and 2-3) to see if the nature of the solvent would affect deuterium content. For the solvents studied, the choice of solvent had little or no effect. Deuteration of the THP ether of 2-pentynol (2-4) gave a product with 97% d_2, about 4% more than with 2-pentynol. With 2-hexynol and its THP ether (2-5 and 2-6), a difference of about 6% in d_2 content was obtained. With 2-pentynol and 2-hexynol it was necessary to add three separate 100 mg portions of Lindlar's catalyst before the reduction proceeded. Quinoline was not used in 2-1 and 18% overreduction to the saturated compound occurred.

Deuteration with Lindlar's catalyst of 3-hexynol and its THP ether (2-7 and 2-8) gave di-deuterated products differing by less than 4% in d_2 content. With the mono- and di-THP ethers of 3-hexyne-1,6-diol (2-9 thru 2-11), no difference was observed in percent d_2 in the products. Entries 2-9 and 2-10 also illustrate that there was no solvent influence on the percent d_2 obtained.

In summary, a high percentage of d_4 incorporation was

TABLE 1

Deuterium Content After Catalytic Deuteration with Wilkinson's Catalyst[a]

Compound number	Compound	Number of deuteriums per molecule (%)							Avg. no. *d.* atoms per molecule
		0	1	2	3	4	5	6	
1-1	CCC≡CCCOH	0.1	0.1	1.1	4.0	91.5	2.9	0.1	3.96
1-2	CCC≡CCCOTHP	0.1	0.1	0.1	2.8	95.9	1.0	0.1	3.98
1-3	THPOCCC≡CCCOH	0.2	0.3	0.0	3.3	94.9	1.2	0.2	3.97
1-4	THPOCCC≡CCCOTHP	0.1	0.2	0.0	2.7	96.5	0.3	0.2	3.97
1-5	CCCC≡CCOH	0.1	0.1	1.8	16.9	78.2	2.9	0.0	3.82
1-6	CCCC≡CCOTHP	0.0	0.0	0.1	3.0	93.8	3.0	0.1	4.00
1-7	CCC≡CCOH	0.1	0.3	2.3	15.6	69.0	12.2	0.5	3.92
1-8	CCC≡CCOTHP	0.1	0.1	0.2	4.3	92.0	3.3	0.1	3.98

[a]Benzene as solvent.

TABLE 2

Deuterium Content After Catalytic Deuteration with Lindlar's Catalyst[a]

Compound number	Compound	Number of deuteriums per molecule (%)							Avg. no. *d.* atoms per molecule
		0	1	2	3	4	5	6	
2-1	CCC≡CCOH	0.0	7.0	92.4	0.6	0.0	0.0	0.0	1.94
2-2	CCC≡CCOH	0.3	5.1	94.1	0.5	0.0	0.0	0.0	1.95
2-3	CCC≡CCOH	0.1	5.8	93.6	0.4	0.0	0.0	0.0	1.95
2-4	CCC≡CCOTHP	1.2	0.8	97.0	0.8	0.0	0.0	0.2	1.98
2-5	CCCC≡CCOH	0.7	6.1	92.9	0.2	0.1	0.0	0.0	1.93
2-6	CCCC≡CCOTHP	0.1	1.0	98.8	0.2	0.0	0.0	0.0	1.99
2-7	CCC≡CCCOH	0.8	4.0	94.7	0.5	0.1	0.0	0.0	1.95
2-8	CCC≡CCCOTHP	0.1	1.6	98.3	0.1	0.0	0.0	0.0	1.98
2-9	THPOCCC≡CCCOH	0.0	1.7	98.0	0.3	0.0	0.0	0.2	1.99
2-10	THPOCCC≡CCCOH	0.0	2.0	97.5	0.3	0.0	0.0	0.2	1.99
2-11	THPOCCC≡CCCOTHP	0.1	3.1	96.7	0.1	0.0	0.0	0.0	1.97

[a]Solvents: benzene, 2-3; diethyl ether, 2-2 and 2-9; hexane, others.

achieved in deuteration with Wilkinson's catalyst of a 3-alkynol, its THP ether or of the THP ether of a 2-alkynol. With the 2-alkynols, however, there was extensive scatter of the deuterium atoms. With the Lindlar catalyst, the position of the triple bond in both the alkynols and their THP ethers had little or no effect on deuterium content.

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ARTICLE

Purification and Characterization of an Extracellular Lipase from the Fungus *Rhizopus delemar* [1]

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The complete purification and characterization of an extracellular lipase (acylglycerol acylhydrolase, EC 3.1.1.3) from *R. delemar* is described. The final product was homogeneous as judged by electrophoresis in denaturing polyacrylamide gels and by isoelectric focusing, and was shown by means of an activity stain to be lipolytic. The purified enzyme had a monomer molecular weight of 30,300, an isoelectric point of 8.6, and approximately one monosaccharide moiety per molecule. *N*-Terminal sequence data (28 residues) and the amino acid composition of the lipase indicated that it corresponds to the product of a lipase-encoding cDNA previously isolated from *R. delemar*. Optimal activity occurred between pH 8.0 and 8.5. The activity and stability of the enzyme were maximum at 30°C. Divalent cations were required for activity, with barium, calcium and manganese conferring maximum activity. Activation by calcium was maximal at and above 10 mM. The lipase was not inactivated by reducing agents, sodium fluoride or phenylmethylsulfonyl fluoride. It was resistant to *N*-ethylmaleimide, and inactivated by *p*-chloromercuribenzoic acid in a manner which was not reversed by cysteine.
***Lipids* 27, 571-576 (1992).**

In biological systems, lipases (acylglycerol acylhydrolases, EC 3.1.1.3) initiate the catabolism of fats and oils by hydrolyzing the fatty acyl ester bonds of acylglycerols. Work from several laboratories has established that the activities of lipases are not limited to this reaction. They also catalyze the synthesis and transesterification of glyceride (1-4) and phosphoglyceride (5,6) ester bonds, and the synthesis and hydrolysis of a variety of non-glyceride esters (7-12). In addition, lipases are active in both aqueous and non-aqueous solvent systems (1,4,10,11,13,14). It has therefore become evident that lipases have considerable biotechnological potential for the general synthesis and hydrolysis of esters (13-17).

Lipases differ from one another in their physical properties and biochemical features, such as substrate specificities, optimal reaction conditions, requirements for activators, and sensitivity to inhibitors (18,19). The mycelial fungus *Rhizopus delemar* produces at least three extracellular lipases (20, 21) which exhibit very high selectivity for the hydrolysis of primary, but not secondary, esters (22). Partially purified mixtures of these enzymes have been employed in a variety of reactions including ester (23) and glyceride synthesis (4,24,25), glyceride restructuring (2,26), and the exchange of the acyl groups of phospholipids (6).

The development of new uses for lipases as applied catalysts would be fostered by their purification and characterization. We report here the purification of one of the extracellular lipases of *R. delemar*. This enzyme corresponds to the product of a lipase-encoding (*LIP*) cDNA recently isolated from this organism (27).

MATERIALS AND METHODS

Enzyme production. Rhizopus delemar, ATCC 34612, was obtained from the American Type Culture Collection (Rockville, MD). The progeny of single spores were used as inocula for enzyme production. Growth medium contained 0.5% Casamino Acids (Difco, Detroit, MI), 30 mM glycerol, trace elements (28) and 5 μg biotin/L in a basal salts medium (29). Media were inoculated with 0.1% (vol/vol) of spore suspension (10^6 spores/L) and shaken at 150 rpm for 36 h at 30°C. The mycelia were then removed by filtration through cheesecloth, and the filtrates were stored at −20°C.

Enzyme purification. Culture filtrate was thawed in two and three liter batches and filtered through Whatman #1 paper. Sodium azide was added to a final concentration of 0.03% (wt/vol). Protease inhibitors were added to the final following concentrations: Phenylmethylsulfonyl fluoride (PMSF, 0.2 mM), pepstatin (1 μM), leupeptin (1 μM) and ethylenediaminetetraacetic acid disodium dihydrate (EDTA, 1 mM). The solution was applied at room temperature to an oleic acid affinity chromatography column (prepared as described below) with a bed volume of 20 mL at a cross sectional flow rate of 0.4 cm/min. Effluent was collected at intervals during loading and checked for lipase activity. When lipase adsorption fell below 40% of input, the column was chilled to 4°C and washed at a rate of 1 mL/min with: (i) 100 mL of 0.86 M NaCl in Buffer 1 (10 mM sodium phosphate, pH 6.0, containing the same inhibitors as were added to crude enzyme), (ii) 100 mL of Buffer 1, and (iii) a linear gradient of Triton X-100 from 0 to 0.5% in 320 mL of 10 mM sodium phosphate, pH 6.0. Fifteen-mL fractions were collected during the gradient.

Lipase-containing fractions from the affinity chromatography step were pooled and applied at 4°C to a CM-Sephadex C25 column (2.6 × 51.5 cm, Pharmacia LKB, Piscataway, NJ) equilibrated in Buffer 2 (10 mM sodium phosphate, pH 6.0, 1 mM EDTA). The column was washed with 400 mL of Buffer 2, followed by a linear gradient of NaCl from 0 to 0.5 M in 1 L of Buffer 2. The flow rate was 1 mL/min throughout. Fifteen-mL fractions were collected during the NaCl gradient.

A rapid and sensitive assay, wherein the hydrolysis of olive oil by lipase induces a change in the fluorescence of rhodamine B (Sigma, St. Louis, MO), was used to track lipase during purification (30). Two- to 100-μL aliquots of

[1]Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Abbreviations: BME, β-mercaptoethanol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium dihydrate; IEF, isoelectric focusing; kDa, kilodaltons; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; pI, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; PVP, polyvinylpyrrolidone; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; U, units of lipase activity; UV, ultraviolet.

potentially lipolytic samples were applied to an agar-solidified emulsion of olive oil and rhodamine B in a Petri dish. The dish was then incubated at room temperature for 5 to 60 min, whereupon active fractions could be detected by their bright fluorescence under ultraviolet (UV) light.

Synthesis of affinity chromatography resin. Oleic acid (1 mol free acid, Sigma), Affi-Gel 102 (20 mL, Bio-Rad Laboratories, Richmond, CA), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (10 mmol, Bio-Rad) were gently mixed overnight at room temperature in 28 mL of 70% aqueous dimethylformamide. The pH was held at 4.2 by the addition of 1N HCl with a constant titrating pH meter. The resin was washed in a column with 700 mL 80% ethanol, followed by two series of (a) 400 mL 0.1 N NH_4OH, and (b) 400 mL 80% ethanol. These were followed by 400 mL 0.1N NH_4OH, and 400 mL 80% methanol. Finally, the column was washed with 1 L 10 mM sodium phosphate, pH 6.0 containing 0.03% sodium azide. The continuous-service life of this resin exceeded two years.

Electrophoretic methods. 0.1% SDS-12% PAGE was performed according to Laemmli (31). The molecular weight of lipase was determined by reference to the mobilities of proteins of known molecular mass (Dalton Mk VII-L, Sigma).

Electrofocusing was performed on a PhastSystem (Pharmacia LKB) according to the manufacturers' directions using IEF 3-9 PhastGels (Pharmacia LKB) pre-equilibrated in a solution of 4M urea, 0.5% Nonidet P-40, and 4% pH 8-10.5 Pharmalytes (Pharmacia LKB). The isoelectric point (pI) of lipase was estimated by comparison of its mobility to those of standard proteins (High pI Calibration Kit, Pharmacia LKB) included on each gel.

Proteins were detected in gels by silver staining (32). Isoelectric focusing (IEF) gels were incubated 5 min in 20% trichloroacetic acid before staining, to eliminate interference by ampholytes.

Lipase activity was detected following IEF by placing the gel onto a solid lipase detection medium (30) containing olive oil and rohdamine B, incubating at room temperature for 100 min, and examining the medium under UV light for bright pink bands.

Determination of lipase activity. Lipolytic activity was quantitated by a continuous titrimetric assay using emulsified olive oil as substrate. A maximum of 0.58 mL of enzyme was added to 5 mL of pre-neutralized emulsion containing 18% (wt/vol) olive oil, 4.2% (wt/vol) gum arabic and 15 mM $CaCl_2$. A titrating pH meter (Radiometer, Copenhagen, Denmark) was employed to maintain the pH at a preset value by the addition of 0.1 N NaOH. Unless stated otherwise, incubations were carried out at 26°C with a set point pH of 7.5. Enzyme activity was calculated from the maximal rate of base addition, assuming molar equivalence between fatty acid release and base consumption. A unit of activity was defined as the release of one μmole of fatty acid per min under these conditions.

Purified lipase was dialyzed against deionized water prior to characterization. In determinations of the pH dependence of activity, the set point pH of the pH meter was adjusted to the values of interest. A thermostatted, water-jacketed reaction chamber was employed in determining the temperature dependence of activity. The effect of storage temperature on activity was measured by preincubating the enzyme at the desired temperature and assaying residual activity at 26°C. For investigations of the effect of cations upon lipase activity, 0.5% (wt/vol) polyvinylpyrrolidone [average mol. wt.: 360 kilodaltons (kDa), Sigma] replaced gum arabic as the emulsifier, and $CaCl_2$ was omitted from the assay mixture.

The calcium content of the gum arabic used as a substrate emulsifier was determined on a Perkin-Elmer (Norwalk, CT) PE 5000 Atomic Absorption Spectrophotometer with reference to $CaCl_2$ solutions of known concentrations.

Protein determination. Protein concentrations were determined according to Bradford (33) using the Bio-Rad Protein Assay Kit. For samples containing Triton X-100, the bicinchoninic acid assay (34) (Pierce, Rockford, IL) was employed. Bovine-γ-globulin was used as the standard in both assays.

Sugar content determination. The phenol-sulfuric acid method (35) was used to determine the degree of glycosylation of the purified lipase, using mannose as the reference standard. As another means of assessing the degree of glycosylation, individual samples of lipase were treated with endoglycosidase F and with *N*-glycosidase F (both from Boehringer Mannheim, Indianapolis, IN) (36), and examined for an alteration in electrophoretic mobility, relative to untreated lipase, by SDS-PAGE.

Amino acid analysis. Pure lipase was incubated in 5.7 N HCl for 24 h at 110°C. The amino acid compositions of the resulting hydrolysates were determined with a Beckman (Palo Alto, CA) Model 119CL amino-acid analyzer according to the manufacturers' instructions.

Amino acid sequence determination. N-Terminal sequence analysis was performed by automated Edman degradation using instrumentation, reagents and protocols from Applied Biosystems (Foster City, CA). A Model 470A gas-phase protein sequencer equipped with an online 120A PTH-analyzer and a Model 900A Control/Data Analysis Module were employed.

RESULTS AND DISCUSSION

Production and purification of lipase. We have produced and have purified to homogeneity an extracellular lipase from *R. delemar.* Glycerol was the main carbon source for the growth of the organism. In the majority of published papers describing lipase production, triglycerides have been the carbon source. However, there are disadvantages to their use, including media turbidity (which complicates measurement of the growth of the culture by optical methods), the insolubility of the fatty acids released by enzymatic hydrolysis, and the difficulty of removing unhydrolyzed triglyceride from solution prior to enzyme purification. We have found glycerol to be an acceptable carbon source for lipase production, resulting in enzyme levels comparable to those obtained with triglycerides, without the difficulties noted above.

Lipase eluted as a single peak from the oleic acid resin, during the Triton X-100 gradient, at a Triton concentration of approximately 0.25%. The output of two or three affinity chromatography run was routinely combined and taken to the CM-Sephadex step. Lipase eluted from this cation exchange column as a single sharp symmetrical peak at 0.25 M NaCl, the approximate mid-point of the gradient. The results of a typical purification are sum-

TABLE 1

Purification of *Rhizopus delemar* Extracellular Lipase

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (-fold)
Filtered culture (22.8L)	350	259,208	741	1
Oleic acid affinity chromatography	19.7	97,926	4971	6.7
CM-Sephadex chromatography	10.1	77,145	7638	10.3

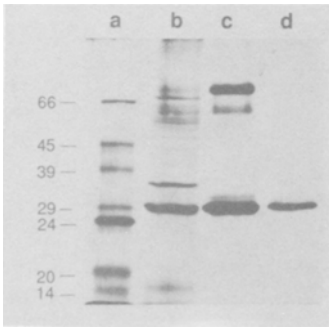

FIG. 1. SDS-PAGE of *R. delemar* lipase at various stages of purification. Proteins were detected by silver staining. Lane a, 2.25 μg of molecular mass marker preparation; lane b, 37.5 μg of culture filtrate; lane c, 3 μg of pooled lipase-positive fractions eluted from oleic acid affinity chromatography column; lane d, 1 μg of pooled lipolytic material obtained by CM-Sephadex column chromatography. The masses of the molecular weight marker proteins (kDa) are indicated in the left margin.

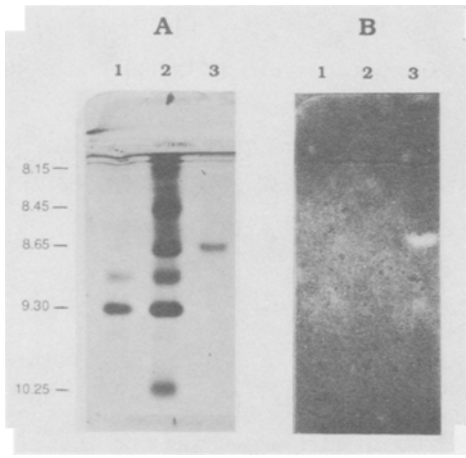

FIG. 2. Isoelectric focusing (IEF) of the purified lipase. (A) Silver stained IEF gel. Lane 1, 0.1 μg of trypsinogen standard; lane 2, 0.433 μg of IEF standard protein preparation; lane 3, 98 ng of pure lipase. The pI values of the standard proteins are indicated in the left margin. (B) Activity stain for lipase. An IEF gel identical to the one in Panel A was inverted onto lipase indicator media containing olive oil and rhodamine B, incubated for 100 min at room temperature, and photographed under UV light. The bright band indicates the site of lipase activity.

marized in Table 1. A 10.3-fold increase in specific activity was achieved, with a 30% recovery of activity. Purified preparations typically had specific activities of approximately 8000 units of lipase activity (U)/mg.

Enzyme characterization. As judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) (Fig. 1) and IEF (Fig. 2A), the final lipase preparation was homogeneous. When an IEF gel was incubated on lipase indicator media, a single fluorescent band was produced (Fig. 2B). The position of this band corresponded to that of the single protein band seen on IEF gels (Fig. 2A). Therefore, the purified protein is a lipase.

The molecular weight of the lipase, calculated by SDS-PAGE, was 30.3 kDa (Fig. 1). The isoelectric point was 8.6 (Fig. 2).

Amino acid composition and N-*terminal sequence.* The amino acid composition of the purified lipase (Table 2), indicates a minimum molecular weight of 29,762 (Trp was not determined). This agrees with the value determined by SDS-PAGE (above). As reported for other lipases, the protein is not enriched in hydrophobic residues, despite the fact that its natural substrates are very hydrophobic.

Two nmol of purified lipase were subjected to *N*-terminal sequence determination. A low background was obtained throughout this operation, indicating that the preparation was very homogeneous. The *N*-terminus was not blocked. The sequence we determined is shown in Scheme 1. An identical sequence is encoded within the *R. delemar* lipase cDNA (27). Based on the gene sequence, the predicted molecular mass for the mature protein with this *N*-terminus is 29,592. This agrees with the value cal-

TABLE 2

Amino Acid Composition of the *R. delemar* Lipase[a]

Amino acid	Determined[b]	Predicted[c]
Ala	19	15
Arg	10	9
Asn	} 28	10
Asp		13
Cys	8	6
Gln	} 24	13
Glu		9
Gly	21	21
His	8	7
Ile	14	17
Leu	17	16
Lys	18	15
Met	2	1
Phe	14	15
Pro	16	15
Ser	22	24
Thr	21	22
Trp	n.d.[d]	3
Tyr	11	12
Val	19	26

[a]Expressed as number of amino acid residues per lipase molecule.
[b]Determined on the purified lipase as described in the text.
[c]Predicted by the nucleotide sequence of the *LIP* cDNA for amino acids +1→ +269 (27).
[d]n.d., Not determined.

1 5 10
H_2N-Ser-Asp-Gly-Gly-Lys-Val-Val-Ala-Ala-Thr-Thr-Ala-Gln-

15 20 25
Ile-Gln-Glu-Phe-Thr-Lys-Tyr-Ala-Gly-Ile-Ala-Ala-Thr-Ala-Tyr-

SCHEME 1

culated for the pure lipase from SDS-PAGE data (above), and with that predicted from the amino acid composition of the enzyme (Table 2). In addition, the amino acid composition of the purified lipase agrees with that predicted from the cDNA sequence (27). We conclude that the lipase described here corresponds to the product of the cloned *LIP* cDNA and that it is initially synthesized as a larger polypeptide which is proteolytically cleaved to produce mature lipase.

The predicted pI of the mature lipase, calculated from the *LIP* cDNA, is 8.01 (27). The difference between this value and the pI determined here for the isolated fungal protein (8.6) may be related to the inclusion of urea and NP-40 in the IEF gel. These additives were absolutely required to detect lipase protein on the gels.

Pure lipase was determined to contain 0.53% carbohydrate. This corresponds to 0.88 monosaccharide residues per molecule. In agreement with this observation, neither endoglycosidase F nor *N*-glycosidase F treatment caused any alteration in the SDS-PAGE mobility of the lipase. In addition, the molecular weight of lipase, determined by SDS-PAGE of either crude (Haas, M.J., unpublished) or purified enzyme (above), is virtually identical to those calculated from the amino acid composition of the enzyme (above) and predicted from the sequence of the cloned lipase gene (27). All evidence therefore indicates that the lipase isolated here lacks substantial glycosylation. By contrast, a glycosylation level of 5–10% appears to be more typical of fungal lipases (37,38). Since the oleic acid affinity chromatography column was run at a low flow rate, this step of the purification required several weeks. It is possible that the lipase was initially glycosylated but that its carbohydrate moieties were removed during this operation by glycosidases endogenous to the crude enzyme preparation.

Properties of the lipase. The pH dependence of the activity of the pure enzyme is shown in Figure 3. Optimal activity occurred at slightly alkaline pH, with peak activity between pH 8.0 and 8.5. The enzyme exhibited 80% or more of maximal activity between pH 7.3 and 9.1. The fact that the lipase is maximally active near its pI may indicate that the binding of this enzyme to its hydrophobic substrate is best when its net charge is zero.

Lipase activity is temperature sensitive (Fig. 4). Maximum activity occurred at 30°C, with 80% of this value exhibited between 25 and 38°C. At and above 45°C, the activity fell to 0 within the first two min of incubation.

The thermal stability (15 min) of the lipase is illustrated in Figure 5. The enzyme was unstable above 30°C. Approximately 50% of the activity was lost during a 15 min preincubation at 42°C. Above 50°C, the enzyme was completely inactivated during a 5-min preincubation.

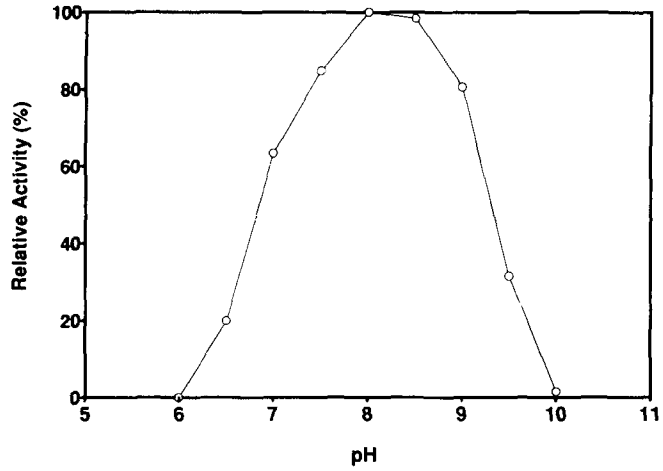

FIG. 3. The effect of pH on the activity of the pure lipase. The activity was assayed titrimetrically at various pH values as described in Materials and Methods, using olive oil as the substrate and gum arabic as emulsifier. Activities are expressed relative to that of the most active sample.

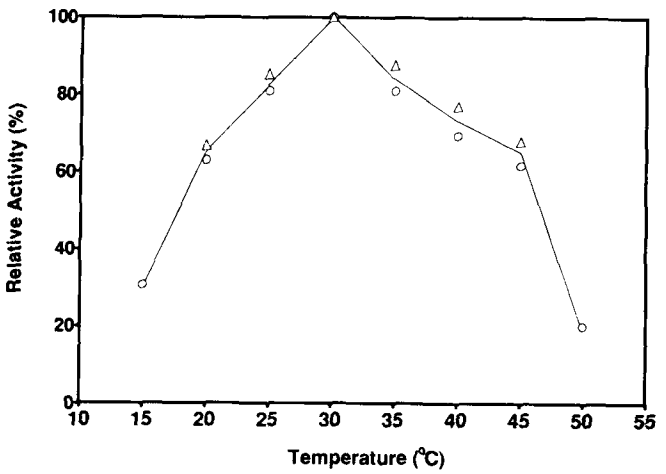

FIG. 4. Dependence of lipase activity upon temperature. Activity was assayed titrimetrically in a thermostatted, water-jacketed reaction vessel as described in Materials and Methods, using olive oil as the substrate and gum arabic as the emulsifier. The results of duplicate determinations are shown. Activities are expressed relative to that of the most active sample.

Some lipolytic enzymes require calcium for activity (18). It has been postulated that this stimulation is mediated through electrostatic interactions which mask repulsions either between the enzyme and its emulsified substrate or between the enzyme and product free fatty acids. To measure the effects of calcium and other ions, lipase activity was assayed in substrate emulsions where gum arabic, which contains inorganic ions, was replaced by the ion-free emulsifier PVP. The effects of the chloride salts of various cations, at concentrations of 10 mM, upon lipase activity were examined. With no additions, lipase activity was undetectable. Barium, calcium and man-

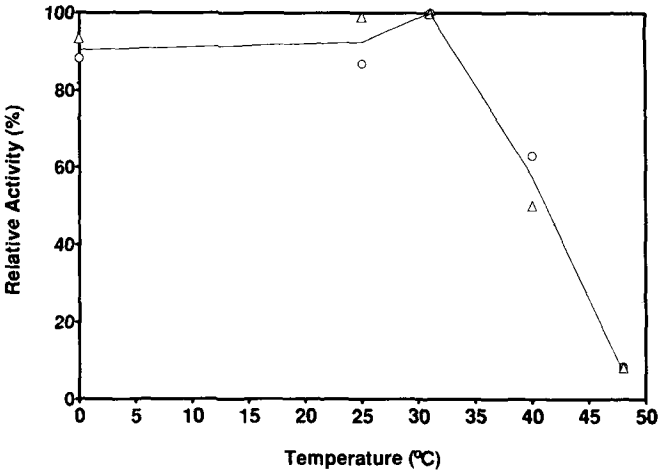

FIG. 5. Thermal stability of the lipase. Aliquots of enzyme were incubated at desired temperatures for 15 min. Lipolytic activity was then assayed titrimetrically at 26°C as described in Materials and Methods. The results of duplicate determinations are shown. Activities are expressed relative to that of the most active sample.

ganese (at 10 mM) substantially restored lipase activity (4400–5300 U/mg). Moderate amounts of activity were recovered in the presence of zinc, cobalt, copper and magnesium (700–3300 U/mg). No activity was detected upon the addition of sodium, potassium or ammonium salts.

The response to calcium was further investigated by testing its effects at several concentrations. Lipase activity rose steadily in PVP-emulsified systems as the calcium concentration increased, and was maximal above 10 mM. Gum arabic contains calcium. By atomic absorption spectroscopy, it was determined that the calcium concentration of gum arabic-emulsified assay mixtures was 68 mM. Nonetheless, the further addition of $CaCl_2$ stimulated the enzyme. Evidently, only a fraction of the calcium introduced by gum arabic is able to effect lipase activity. The stimulation by added $CaCl_2$ was maximal at and above 10 mM, resulting in an approximately three-fold increase in activity over that seen in the absence of added $CaCl_2$.

The effect of enzyme inhibitors upon lipase activity is shown in Table 3. The enzyme is resistant to the disulfide bond reducing agents dithiothreitol (DTT) and β-mercaptoethanol (BME). The predicted amino acid sequence of the *R. delemar* lipase is highly homologous to that of *Rhizomucor miehei*, which contains three disulfide bonds (27,42). The *R. delemar* lipase contains Cys at sites corresponding to the six Cys residues involved in disulfide formation in the *R. miehei* enzyme. It is reasonable to postulate that the *R. delemar* enzyme also has three disulfide bonds. The resistance of the *R. delemar* lipase to inactivation by reducing agents indicates that these disulfides are either inaccessible or are not essential for activity.

The enzyme was resistant to *N*-ethylmaleimide (NEM) (Table 3), consistent with the postulate that its sulfhydryls are involved in disulfide bonds. However, 2 mM *p*-chloromercuribenzoic acid (PCMB) did inhibit the lipase. Activity was only partially restored by cysteine (Table 3), suggesting that PCMB inhibition is due to action at some

TABLE 3

Stability of *R. delemar* Lipase[a]

Treatment	Remaining activity (% of control)
BME, 10 or 25 mM, 64 h, 26°C	94 ± 4
DTT, 10 mM, 0.5 h, 26°C	112 ± 16
NaF, 10 mM, 0.5 h, 26°C	109 ± 2
PMSF, 0.2 mM, 0.5 h, 26°C	105 ± 1
NEM, 10 mM, 4 h, 26°C	101 ± 6
pCMB, 1 mM, 0.5 h, 26°C	87 ± 7
pCMB, 2 mM, 0.5 h, 26°C	5.1 ± 0.9
pCMB, 2 mM, 0.5 h, 26°C + cysteine, 5 mM, 0.66 h, 26°C	51 ± 6

[a]The activities are expressed as the mean and standard deviation of two determinations.

secondary site within the enzyme rather than to an interaction with a sulfhydryl group.

Sodium fluoride inhibits some lipases (39), while others are resistant to its effects (40). The *R. delemar* enzyme was not inhibited by fluoride (Table 3).

A serine-containing pentapeptide sequence has been postulated to form part of the active site of lipases, with the serine playing an active role in catalysis (41). The *R. delemar* lipase gene contains sequences encoding this conserved pentapeptide (27). However, the purified lipase was resistant to phenylmethylsulfonyl fluoride (PMSF), a potent serine-directed inhibitor (Table 3). Similar resistance has been reported for the *R. miehei* lipase (42), and attributed to a protective burying of the active site serine within the enzyme. Presumably this is also the case for the closely related *R. delemar* enzyme.

This is the first description of the complete purification and characterization of a *Rhizopus* lipase for which the cognate gene has also been isolated and analyzed. The size, pI and amino acid composition of the lipase reported here corresponds to those predicted for a peptide encoded by an internal portion of the cloned *LIP* cDNA (27). This correspondence led to the conclusion (27) that the lipase is initially synthesized as a precursor of molecular mass 42.1 kDa which undergoes proteolytic maturation to a smaller species with molecular mass of approximately 30 kDa.

The molecular mass of the *R. delemar* lipase is similar to that of a lipase from *Rhizomucor miehei* (38,42), and about 10 kDa smaller than that reported to date for a *Rhizopus* lipase. Iwai's group reported the partial purification of three extracellular lipases from *R. delemar* (20,21). The enzyme we have isolated is similar to their lipase C in terms of stability and pH and temperature requirements. However, the molecular mass of lipase C, determined by gel filtration, was 45 kDa, 15 kDa larger than the enzyme we have isolated. This larger molecular weight may be an overestimate of the true mass, since gel filtration was observed to overestimate the mass of another lipase, that from *R. miehei* (38). Alternatively, lipase C may have retained an associated glycopeptide during purification, as was demonstrated for *Rhizopus arrhizus* lipase (43–45). This lipase was isolated as a heterodimer consisting of a lipolytic fragment with a molecular mass of approximately 34 kDa, and a non-lipolytic glycopeptide of 8.5 kDa. The two fragments were separated by treat-

ments such as acid precipitation, but this resulted in the inactivation of the lipase (45). Considering the close relationship between members of the genus *Rhizopus* (46), it seems probable that the *R. delemar* lipase is produced in a similar fashion, with the C lipase described by Iwai's group representing the heterodimer form of the enzyme we have isolated. The purification method here appears to allow the preparation of glycopeptide-free lipase which retains activity.

It is conceivable that the glycopeptides attached to the *R. arrhizus* lipases isolated by Laboureur and Labrousse (43) and Semeriva *et al.* (44,45) are lipase propeptides which remain associated with the enzyme after proteolytic maturation. Chiba *et al.* (47) reported the isolation of a lipase from a commercial preparation of crude *R. delemar* enzyme. The molecular weight of the enzyme was 41.3 kDa, similar to those of the enzymes studied by Laboureur and Labrousse (43) and Semeriva *et al.* (44,45). In size this enzyme corresponds to the prolipase encoded by the cloned *R. delemar* lipase gene (27). However, its amino acid composition (47) differs substantially from that predicted for the *R. delemar* prolipase (27). Therefore, it is unlikely that the glycopeptides found with some *Rhizopus* lipases are lipase propeptide fragments which have remained associated with the enzyme after proteolytic maturation of a precursor form of the enzyme.

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Reversible ATP-Dependent Inactivation of Adipose Diacylglycerol Acyltransferase

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Diacylglycerol acyltransferase from rat adipose tissue is shown to be inactivated by 30 to 40% upon incubation with adenosine 5′-triphosphate (ATP) and Mg^{2+}. The activity responsible for this inactivation is associated with the cytosolic fraction, specific for ATP, prevented when ATP is substituted by β,γ-methylene-ATP, and partially blocked by 1 mM ethylenediaminetetraacetate or 40 mM NaF, but not by inhibitors of adenosine 3′,5′-cyclic-monophosphate (cAMP)-dependent protein kinase and/or protein kinase C (PKC). The cytosolic activity cannot be mimicked by (cAMP)-protein kinase nor by PKC. Inactivated diacylglycerol acyltransferase from ATP/cytosol-treated microsomes can be reactivated by incubation with partially purified protein phosphate from rat liver, and can be inactivated again by further addition of ATP in the presence of cytosol. The results suggest the existence in adipose tissue of a protein kinase other than cAMP-protein kinase or PKC, which may be involved in the regulation of triacylglycerol synthesis.
Lipids 27, 577–581 (1992).

Incorporation of fatty acids into adipose tissue triacylglycerols plays a central role in lipid metabolism. Although it is well established that this process is under hormonal control, the precise molecular events involved in the regulation have yet to be elucidated. *In vitro* experiments suggest that some triacylglycerol synthetic enzymes from the heart and the liver may be regulated by a phosphorylation-dephosphorylation mechanism (1–3). We have recently reported the presence of an enzyme activity in adipose tissue which in the presence of adenosine 5′-triphosphate (ATP) inactivates glycerolphosphate acyltransferase (GPAT) (4). A second key enzyme in triacylglycerol synthesis, diacylglycerol acyltransferase (DGAT), is shown here to be reversibly modified by a cytosolic enzyme activity in the presence of ATP and Mg^{2+}.

MATERIALS AND METHODS

Materials. All substrates, nucleotides, fatty acid-free bovine serum albumin (BSA) the catalytic subunit of adenosine 3′,5′-cyclic monophosphate (cAMP)-protein kinase from bovine heart, staurosporine, amiloride and 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine dihydrochloride[1] (H7 analogue) were obtained from the Sigma Chemical Co. (St. Louis, MO). Rat brain protein kinase C was from Calbiochem Co. (San Diego, CA). L-[2-^{3}H]Glycerol-3-phosphate (10.6 Ci/nmol, 1 Ci = 37 GBq) and [1-^{14}C]oleoyl CoA (58 Ci/mol) were from New England Nuclear (Lachine, Quebec, Canada). Universol (liquid scintillation cocktail) was obtained from ICN biomedicals (Mississauga, Ontario, Canada), and Sephadex G-25 was purchased from Pharmacia Inc. (Baie d'Urfe, Quebec, Canada).

Protein phosphatase was partially purified from rat liver up to the ethanol precipitation step of the method by Brant *et al.* (6) for phosphorylase phosphatase purification. The ethanol precipitate was extracted twice with 25 mM Tris-HCl (pH 7.5) buffer containing 1 mM ethylenediaminetetraacetate (EDTA) and 1 mM DL-dithiothreitol (DTT). The extract was then dialyzed against the same buffer, concentrated, added to one volume of glycerol and kept at −90°C.

Epididymal adipose tissue from eight male Sprague-Dawley rats (Charles River Canada Inc., St. Constant, Quebec, Canada), fed *ad libitum*, were homogenized and fractionated as previously described by Walsh *et al.* (4). The 16,000 × *g* supernatant (postmitochondrial supernatant) was resolved into cytosol and microsomes by centrifugation at 100,000 × *g* for 1 h. The microsomes were washed once and resuspended in a 10 mM Tris-HCl (pH 7.5) buffer containing 0.25 M sucrose and 1 mM DTT to a final concentration of 1–2 mg protein/mL. Both fractions were stored in aliquots at −90°C. Amounts of microsomes used in the experiments are indicated through this paper as mass of microsomal protein.

Methods. DGAT was assayed as previously described (7) by incubating the microsomes (10–20 μg) for 6–8 min at 37°C with 20 μM [1-^{14}C]oleoyl-CoA (10 Ci/mol) and 150 μM 1,2-dioleoyl-*sn*-glycerol (delivered in 5 μL acetone) in 0.5 mL of 0.1 M Tris-HCl (pH 7.5), containing 1 mM DTT, 10 mM $MgCl_2$ and 5 μM BSA (TMB Buffer). The reaction was stopped by the addition of 1.5 mL of isopropanol/heptane (1:1, vol/vol). The organic phase was washed three times with 0.75 mL of isopropanol/heptane (4:1, vol/vol) and 0.75 mL of 0.05% KOH. A 0.75-mL aliquot from the organic (heptane) phase was counted in 7.5 mL of Universol in an LKB-Wallac (Turku, Finland) 1215 Rack Beta counter.

Glycerolphosphate acyltransferase (GPAT) was assayed as previously described (4). NADPH-cytochrome reductase was assayed with cytochrome-C as described by Phillips and Langdom (8). All enzyme assays were performed in duplicate. Protein concentrations were measured by the dye binding method of Bradford (9), using BSA as a standard.

ATP-treated microsomes were prepared by incubation of microsomes (0.4 mg/mL) with 0.4 mg/mL cytosol, 1 mM ATKP, 10 mM Mg^{2+}, and 2 mM DTT in 100 mM Tris-HCl (pH 7.5) for 10 min at room temperature. ATP was then removed in a Sephadex G-25 (fine) column (4 mL

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[1]This compound was mistakenly listed by Sigma as H7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine) and found to be a PKC inhibitor, although less potent than H7 (5).

Abbreviations: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-phosphate; AMP-PCP, β,γ-methylene-ATP; ATP, adenosine 5′-triphosphate; BSA, fatty acid-free bovine serum albumin; cAMP, adenosine 3′,5′-cyclic monophosphate; Cl-Ph-S-cAMP, 8-(4-chlorophenylthio)-cAMP; CTP, cytidine 5′-triphosphate; DGAT, diacylglycerol acyltransferase; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetate; GPAT, glycerolphosphate acyltransferase; GTP, guanosine 5″triphosphate; H-7 analogue, 1-(5-isoquinolinylsulfonyl)-3-methylpiperazine dihydrochloride; ITP, inosine 5′-triphosphate; PKC, protein kinase C; PPase, protein phosphatase; UTP, uridine 5′-triphosphate.

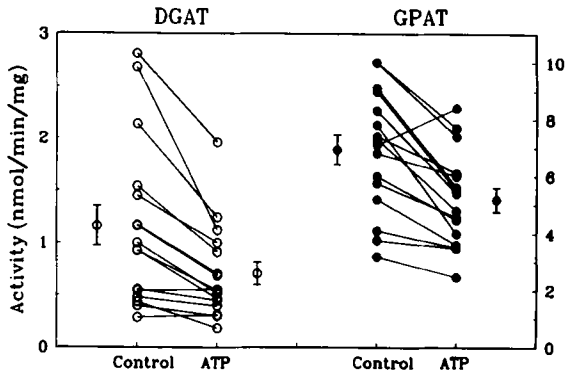

FIG. 1. Postmitochondrial supernatant inactivation with ATP. Adipose tissues from 16 animals were homogenized and 100 μL of the postmitochondrial supernatants (300–600 μg) were incubated for 15 min at 37°C with and without 1 mM ATP in 0.25 mL of 0.1 M Tris-HCl pH 7.5) buffer containing 2 mM DTT, 20 mM $MgCl_2$ 10 μM BSA (50 μM for GPAT). After preincubation, substrates were added (in 250 μL of 0.1 M Tris-HCl) for DGAT (○) and GPAT (●) activity determination as described in Methods.

(4 mL gel/mL sample) spun at 400 × *g* for 1 min in an IEC (Needham Heights, MA) HN-S11 centrifuge. NADPH-cytochrome reductase activity was used as an internal standard for evaluation of microsomal recovery.

RESULTS

Figure 1 illustrates the consistent decrease in both DGAT and GPAT activities observed when post-mitochondrial supernatant from rat adipose tissue was incubated with ATP. GPAT activity (6.9 ± 0.5 nmol•min^{-1}•mg^{-1}) showed a significant reduction of 25 ± 3% (n = 16, $p < 10^{-6}$, paired *t*-test), which correlates well with our previous results (4). DGAT activity (1.2 ± 0.2 nmol•min^{-1}•mg^{-1}) on the other hand, showed a slightly higher degree of inactivation: 35 ± 4% (n = 16, $p < 10^{-7}$). Two animals did not follow the general trend and showed increases of 7 and 1% for DGAT activity while GPAT activity increased by 12 and 26%, respectively. One animal also showed an increase in GPAT activity (18%) with a concurrent decrease in DGAT activity (58%). No statistical correlation was found between GPAT and DGAT inactivation. Addition of 50 mM NaF to the homogenization buffer resulted in no change of either GPAT or DGAT specific activities nor in the ATP-dependent inactivation.

Upon fractionation of the postmitochondrial supernatant into microsomal and cytosolic fractions, the activity responsible for DGAT inactivation was found to be associated with the soluble fraction (Table 1) as previously observed for GPAT inactivation (4). In the absence of cytosol, microsomes from different preparations showed no significant inactivation of DGAT activity. By contrast, in the presence of the cytosolic fraction, a significant reduction of 33 ± 2% in DGAT activity was observed. The degree of DGAT inactivation varied from 28 to 45% and depended on both the cytosol and the microsomal preparations.

As previously demonstrated for GPAT activity (4), microsomal DGAT is significantly stimulated by the cytosolic fraction from 4.3 ± 0.7 to 7.3 ± 0.8 nmol•min^{-1}•mg^{-1} ($P < 0.04$; Table 1). The cytosolic effect on both DGAT activity and its ATP-dependent inactivation is concentration dependent (Fig. 2). Higher concentration of cytosolic protein seems to be required for maximal DGAT inactivation than for stimulation. Boiling of the cytosol for 5 min abolished both of these effects (not shown).

Results in Table 1 and Figure 2 may suggest that the ATP effect on DGAT activity could be due to prevention of cytosolic stimulation of DGAT. However, this assumption does not explain the results in Figure 1, nor the observation that some cytosolic preparations show no capacity for DGAT stimulation while still being able to inactivate the enzyme in the presence of ATP. Preincubation of microsomes with cytosol previous to the addition of ATP does not affect the degree of inactivation. Moreover, preincubation of the cytosol with ATP and Mg^{++} does not modify its capacity for both stimulation and ATP-dependent inactivation of DGAT activity (not shown), suggesting that ATP does not inactivate a cytosolic stimulatory factor.

In contrast with what we observed for GPAT inactivation (4), ATP-dependent inactivation of DGAT did not display a lag time and was detectable after a one-minute incubation (Fig. 3). Therefore, the cytosolic activity responsible for DGAT inactivation appears to have a substantially higher rate than DGAT itself.

We investigated the nucleotide specificity of the cytosolic activity responsible for DGAT inactivation (Table 2) and the results paralleled our earlier findings for GPAT (4). The specificity is such that ATP cannot be substituted by any of the other nucleotides tested. AMP-PCP (β,γ-methylene-ATP) was not a substrate for the DGAT inactivation, suggesting that cleavage of the β,γ-phosphodiester bond of ATP is involved in the reaction. Maximal effect of ATP was observed at concentrations of 0.5 mM or higher (not shown).

The effect of several kinase inhibitors on the ATP-

TABLE 1

Cytosolic Contribution to DGAT Inactivation[a]

Microsomes/cytosol	DGAT (nmol•min^{-1}•mg^{-1})		Inactivation	
	Control	ATP	(%)	p^b
yes/no	4.3 ± 0.7 (9)[c]	4.2 ± 0.7 (9)	3 ± 8	n.s.
yes/yes	7.3 ± 0.8 (25)	4.8 ± 0.5 (25)	33 ± 2	$<10^{-6}$

[a]Microsomes (20 μg in average) alone or with cytosol (100 μg in average) were incubated (in duplicate) for 10 min at 37°C in 0.4 mL of TMB buffer followed by addition of substrates for DGAT assay. n.s., Not significant.
[b]For paired *t*-test.
[c]Number of independent experiments.

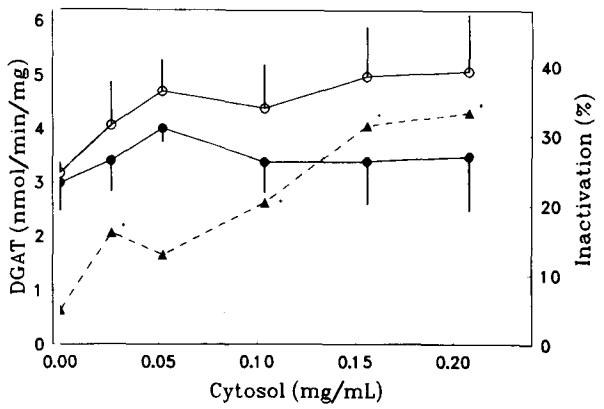

FIG. 2. Cytosol dependence of DGAT inactivation. Microsomes (15–20 μg protein) were incubated for 10 min at 37°C with increasing amounts of cytosol in the presence (●) and absence (○) of 1 mM ATP in 0.4 mL of TMB buffer containing 60 mM sucrose, 0.25 mM EDTA. Following addition of substrates, DGAT activity (left axis) was determined as described in Methods, and percent DGAT inactivation calculated for each cytosol concentration (▲, right axis). Four independent experiments were performed. DGAT activity values are given as the means with their SE. Percent DGAT inactivation values were calculated for each experiment and analyzed by paired *t*-test for significant inactivation. Points represents means values of inactivation and were labeled with * when $P < 0.05$.

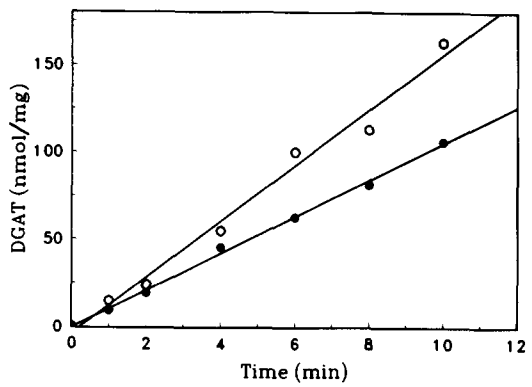

FIG. 3. Time dependence of DGAT inactivation. Microsomes (14 μg in average) with added cytosol (70 μg in average) were assayed for DGAT activity at different incubation times in the presence (●) and absence (○) of 1 mM ATP. Results represent the average of two independent experiments and are fitted by linear regression.

dependent inactivation of DGAT (Table 3) was tested at a non-saturating concentration of ATP (0.2 mM). Partial blockage of the inactivation was achieved only when the incubation mixture contained 1 mM EDTA (with no Mg^{2+}) or 40 mM NaF. Although amiloride seemed to have an effect, statistical analysis indicated no significant difference from the control. Stimulators of cAMP-dependent protein kinases, such as cAMP and 8-(4-chlorophenylthio)-cAMP, did not increase the degree of DGAT inactivation. Moreover, substitution of cytosol by the catalytic subunit of the cAMP-protein kinase or protein kinase C (PKC), did not result in a significant inactivation by ATP: 17 ± 6.3% (n = 4, $P > 0.1$) and −4 ± 15% (n = 4, $P > 0.4$), respectively *vs.* 13 ± 7.1% (n = 4, $P > 0.05$) for the microsomes alone.

As observed for GPAT, we were unable to reactivate the

TABLE 2

Nucleotide Specificity[a]

Nucleotide	DGAT (%)	*P*
—	100	
ATP	62 ± 4.0 (7)	$<10^{-8}$
ADP	82 ± 7.8 (6)	0.07
AMP	104 ± 16 (4)	0.41
GTP	84 ± 8.1 (5)	0.12
CTP	110 ± 16 (4)	0.57
UTP	101 ± 15 (4)	0.95
ITP	103 ± 9.4 (4)	0.77
AMP-PCP	92 ± 7.9 (4)	0.38

[a]Microsomes (18 μg in average) with added cytosolic fraction (100 μg in average) were incubated for 10 min at 37°C in 0.4 mL of TMB buffer in the presence of 1 mM of the indicated nucleotides and assayed for DGAT activity (in duplicate). Results are the mean ± SEM for the indicated number (in bracket) of independent experiments, and are expressed as a percentage of the activity in the control with no nucleotide (9.5 ± 0.7 $nmol \cdot min^{-1} \cdot mg^{-1}$). The *P* values correspond to paired *t*-test with the control as reference.

TABLE 3

Effect of Protein Kinase Inhibitors and Activators[a]

None	Inactivation (%)	*P*
None	29 ± 2.4 (12)	—
EDTA (1 mM)[b]	15 ± 6.5 (4)	0.014
NaF (40 mM)	11 ± 11 (4)	0.024
H7 analogue (0.5 mM)	23 ± 6.5 (5)	0.27
Staurosporine (40 μM)	23 ± 5.5 (5)	0.24
Amiloride (2 mM)	19 ± 6.8 (4)	0.09
cAMP-PK Inhibitor (0.1 mg)	29 ± 9.3 (4)	0.96
cAMP (10 μM)	30 ± 10 (4)	0.92
Cl-Ph-S-cAMP (10 μM)	28 ± 14 (3)	0.87

[a]Microsomes (18 μg in average) with added cytosol (100 μg in average) and the indicated inhibitor/activator were incubated in the presence and absence of 0.2 mM ATP as for Table 1, and assayed for DGAT activity. The corresponding decrease in DGAT activity caused by ATP is given as mean ± SEM for the number of independent experiments indicated in brackets. The *P* values were calculated using a *t*-test with the control (no inhibitor/activator) as reference.

[b]With no Mg^{2+} in the incubation buffer.

ATP-treated microsomal DGAT by incubation with either adipose tissue or liver cytosols and Mg^{2+}. However, reactivation was achieved by incubation with a crude preparation of protein phosphatase from the liver (Fig. 4). The reactivation effect is Mg^{++} dependent and is reduced by 70% in the presence of 40 mM NaF but it is insensitive to 0.1 μM okadaic acid (Rodriquez, M.A., and Lay, T.E., unpublished results).

The reversibility of the phosphatase-dependent DGAT reactivation was also investigated. ATP/cytosol-treated microsomes incubated for 10 min at 37°C in the presence of the phosphatase resulted in a 118 ± 21% reactivation. Another 10-min incubation at 37°C upon addition of cytosol and 1 mM ATP resulted in a 45 ± 4% inactivation, or a 83% reversion of the reactivation (average of six independent experiments). Our liver protein phosphatase preparation was also able to stimulate DGAT activity in untreated microsomes (144 ± 25%, n = 10) and the effect was significantly ($P < 0.02$, by paired *t*-test) reduced by the adipose cytosol (stimulation of 99 ± 27%, n = 10).

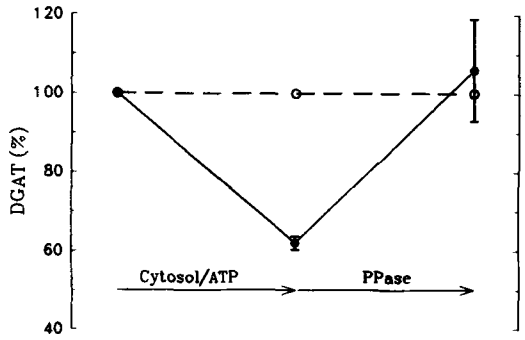

FIG. 4. Reactivation of ATP treated microsomes. Microsomes (0.36 mg/mL) and cytosol (0.36 mg/mL) were incubated in TMB buffer for 10 min at 21°C in the presence of 1 mM ATP, followed by centrifugation on Sephadex G-25 and DGAT determination as described in Methods. Partially purified protein phosphatase from rat liver was added to the remaining filtrate (0.7 mg/mL) and incubated again for 10 min at 37°C, then assayed for DGAT activity. A control (○) was run in parallel without ATP. DGAT activity in the sample (●) is expressed as percentage of the control. Results are the mean ± SEM for three independent experiments. PPase, protein phosphatase.

DISCUSSION

Our results indicate the presence in adipose tissue of an ATP-dependent activity that *in vitro* reduces both glycerolphosphate acyltransferase and diacylglycerol acyltransferase activities by 30 to 40%. As previously shown for GPAT inactivation (4), the activity responsible for DGAT is associated with the cytosolic fraction, is heat sensitive, requires Mg^{2+} and an intact, cleavable β,γ-phosphodiester bond on the ATP. DGAT activity from ATP-treated microsomes can be restored to control levels by incubation with a crude preparation of liver protein phosphatase. New addition of ATP and cytosol results in reinactivation close to the level for ATP-treated microsomes. These observations support the hypothesis of a soluble protein kinase involved in the regulation of adipose tissue DGAT, as has been suggested for microsomal GPAT (4).

DGAT inactivation was not affected by the inhibitors of PKC and/or cAMP-dependent protein kinase such as H-7 analogue (5), staurosporine (10), amiloride (11,12) or cAMP-protein kinase inhibitor. Furthermore, since the stimulation of DGAT was not observed by the addition of Ca^{2+}, cAMP or the cAMP analogue chlorophenylthio-cAMP, and because neither PKC from rat brain nor the catalytic subunit of cAMP-dependent protein kinase from bovine heart could replace the cytosolic activity, the postulated DGAT kinase is neither PKC nor cAMP-protein kinase.

Modulation of DGAT activity in liver by a phosphorylation-dephosphorylation mechanism was suggested previously by Haagsman and coworkers (3,13). Rat liver microsomes were shown to be inactivated *in vitro* by incubation with ATP, Mg^{++} and the liver cytosolic fraction, but not with cAMP-protein kinase and its substrates (3). The authors reported reactivation of DGAT activity from ATP-trated liver microsomes upon incubation with liver cytosol. Also they observed a decrease in DGAT activity of tissue homogenized in the presence of 40 mM NaF. By contrast, we were unable to detect either of these latter effects in adipose tissue. We did find a consistent stimulation of both DGAT (Table 1) and GPAT (4) activities by the cytosolic fraction. Similar stimulation has been previously reported in different laboratories although the mechanism of stimulation has not yet been demonstrated (14-17).

Regulation of adipocyte microsomal GPAT by cAMP-protein kinase has been suggested by Nimmo and coworkers (18-20), but other laboratories have been unable to observe any GPAT inactivation upon incubation of rat adipocyte microsomes with cAMP-protein kinase and its substrates (4,21). We have consistently observed cytosol and ATP-dependent inactivation of GPAT.

Inactivation of both DGAT and GPAT enzymes show similar nucleotide dependence and specificity, as well as similar response to protein kinase inhibitors. Although inactivation of both enzymes cannot be reversed by the addition of cytosol alone, they can both be reversed by our preparation of protein phosphatase from liver. Whether or not GPAT and DGAT are substrates of the same protein kinase remains to be established.

Since DGAT in ATP and cytosol-treated microsomes can be reversibly reactivated by our crude preparation of phosphorylase phosphatase from liver, and since this reactivation is dependent on Mg^{++} and sensitive to NaF, a protein phosphatase (likely PPase 2C) is implicated in the process. The ability of the protein phosphatase preparation to stimulate DGAT activity of untreated microsomes indicates that the DGAT enzyme is already in a partially inactivated form. Prevention of the latter stimulation by the adipose cytosol, and the failure of cytosol to reactivate ATP-treated microsomes may both be direct results of the reported phosphatase inhibitors present in adipose tissue (19,22). Consequently, future experiments will try to resolve the adipose tissue protein phosphatase from these inhibitors.

ACKNOWLEDGMENTS

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Reversible or Irreversible Modification of [^{3}H]PAF Binding on Rabbit Platelet Membranes Differentiates Various PAF Receptor Antagonists[1]

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[^{3}H]Platelet-activating factor (PAF) binding to rabbit platelet membranes was examined before and after 20 min preincubation at 25°C in the presence of PAF, lysoPAF, or of five different PAF receptor antagonists (L 652731, BN 52021, WEB 2086, BN 52111 and BN 52115). When platelet membranes were not washed after preincubation with PAF or PAF antagonists, no significant specific binding of [^{3}H]PAF was observed, which suggests full occupancy of the binding sites. When membranes were extensively washed, full recovery of specific [^{3}H]PAF binding was attained with L 652731 and partial recoveries (60%, 55% and 30%) were reached with BN 52021, WEB 2086 and PAF, respectively; no recovery was seen with the dioxolanes BN 52111 and BN 52115. Scatchard analysis of the binding data indicated that no significant change in the dissociation constant (K_d) and maximum number of binding sites (B_{max}) occurred after preincubation of platelet membrane with L 652731, whereas a reduction of B_{max} was observed when PAF and BN 52021 were measured. When platelet membranes were preincubated with WEB 2086, B_{max} and K_d significantly increased. The data suggest differing binding properties for PAF and the PAF antagonists. Some of the PAF antagonists may tightly bind to the PAF receptor site(s) and/or irreversibly modify or downregulate PAF recognition sites. Our results also suggest that the interaction of PAF receptor antagonists with PAF receptor can be divided into at least two components, namely a reversible component and an irreversible one. *Lipids 27*, 582–586 (1992).

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycerophosphocholine) (1,2) is a biologically active phospholipid released by a variety of cell types, such as macrophages, leukocytes, basophils, platelets and endothelial cells, upon appropriate stimulation. PAF appears to be involved in inflammatory disorders in lung (3,4), heart (5,6) and other organs (7,8). The structural requirements for PAF biological activity are highly specific, which led to the notion that PAF action is mediated through specific receptor(s).

Specific binding for [^{3}H]PAF has been described in many organs and cell types including platelets (9–11), lung tissue (12), polymorphonuclear neutrophils (13,14), endothelial cells, liver (15), brain (16,17) and the eyes (18). Different characteristics for the PAF binding sites in these tissues have been described, which suggests existence of multiconformational states of one type of receptor or populations of multiple receptors (15). [^{3}H]PAF binding has been shown to be specific, saturable and displaceable by unlabeled PAF (10) or PAF receptor antagonists, including CV 3988 (19), L 652731 (20), WEB 2086 (21), BN 52111, BN 52115 (22) kadsurenone (23) and BN 52021 (24).

[^{3}H]PAF binding to intact human platelets is prevented by preincubation of platelets with unlabeled PAF, suggesting a downregulation of the PAF binding sites (25,26). We further investigated the effect on [^{3}H]PAF binding of preincubation of rabbit platelet membranes with increasing concentrations of PAF and five different PAF-receptor antagonists structurally unrelated to PAF. These antagonists include: The terpenoid BN 52021, the tetrahydrofurane L 652731, the triazolothienobenzodiazepine WEB 2086 and two PAF-related molecules, BN 52111 and BN 52115.

MATERIALS AND METHODS

Materials. Synthetic [^{3}H]PAF in ethanol with a specific activity of 59.5 Ci/mmole was purchased from New England Nuclear (Boston, MA). Unlabeled PAF and lysoPAF were purchased from Calbiochem (San Diego, CA) or Bachem (Bubendorf, Switzerland), solubilized in ethanol and stored at −80°C. BN 52021 was solubilized in dimethylsulfoxide (DMSO). L 652731 and WEB 2086, kindly provided by Merck Sharp and Dohme (St. Louis, MO) and Boehringer Ingelheim (Ingelheim/Rhein, Germany), were also solubilized in DMSO. BN 52111 and BN 52115 were solubilized in water. The chemical structures of the compounds are given in Figure 1.

Preparation of platelet membranes. Rabbit blood (6 vol) was collected from the central ear artery and added to 1 vol of ACD solution (citric acid 1.4 g, sodium citrate 2.5 g, dextrose 2 g per 100 mL of distilled water). After centrifugation at 150 *g* for 15 min, the platelet-rich plasma (PRP) was carefully removed and centrifuged again for 15 min at 1000 × *g*. The platelet pellet was collected and successively washed by centrifugation in i) a 37°C prewarmed Tyrode buffer containing heparin (50 U/mL), apyrase (0.3 U/mL) and ethylenediaminetetraacetic acid (EDTA; .2 mM); ii) 10 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, pH 7.4 buffer; and iii)the latter buffer sodium-free at pH 7. The platelet pellet was then resuspended in the latter buffer, quickly frozen in liquid nitrogen and slowly thawed at room temperature; the whole process was repeated at least three times as described by Hwang *et al.* (27). The lysed platelets were centrifuged at 100,000 × *g* for 30 min in a Beckman (Palo Alto, CA) model L8-55 ultracentrifuge (50.2 Tirotor). The platelet membrane homogenate was stored at −80°C and used within 5 days. Protein content was determined by the Bradford method (28) using Biorad (Paris, France) protein assay reagents and bovine serum albumin (BSA) as a standard.

Preincubation of platelet membranes with PAF, lysoPAF and PAF-antagonists. For the preincubation pro-

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

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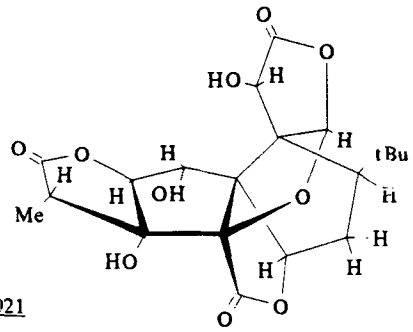
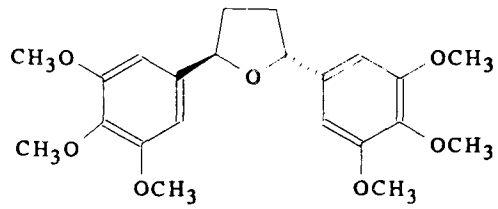
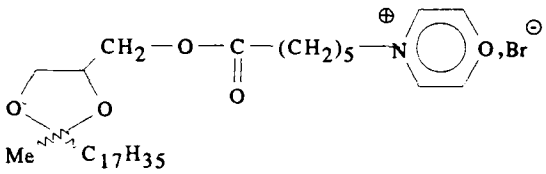
Abbreviations: BSA, bovine serum albumin; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PMSF, phenylmethylsulfonyl fluoride, PRP, platelet-rich plasma.



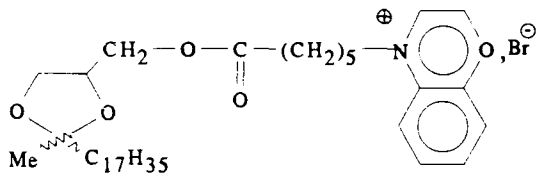
BN 52021



L 652731



BN 52111



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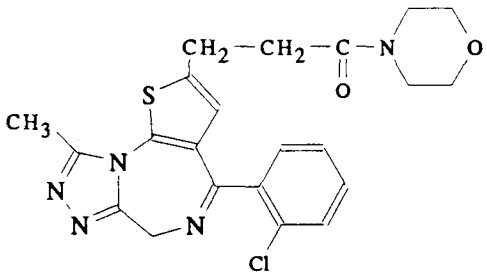

WEB 2086

FIG. 1. Chemical structures of L 652731, BN 52021, BN 52111, BN 52115 and WEB 2086.

cedure, the membrane homogenate was suspended in a solution containing 10 mM Tris, 5 mM $MgCl_2$, 2 mM EDTA, 10^{-4} M phenylmethylsulfonyl fluoride (PMSF), trasylol (100 U) per mL and BSA 0.025% at pH 7 (buffer A). The membrane homogenate was then preincubated for 20 min at 25°C in presence or in the absence of lysoPAF (10^{-6} M), PAF (10^{-6} M) or a PAF antagonists (10^{-5} M).

After preincubation, the membranes were centrifuged at 100,000 × *g* for 40 min in Buffer A. The pellet was washed five times by centrifugation at 100,000 × *g* for 30 min in the same buffer. Finally, the platelet pellet was suspended in Buffer A and used for the binding assay.

[^{3}H]PAF binding assay. One hundred to 150 μg of membrane protein was added to a final volume of 1 mL of 10 mM Tris, 5 mM $MgCl_2$, BSA 0.025% pH 7 buffer containing 1 nM [^{3}H]PAF and was incubated for 90 min at 0°C with or without unlabeled PAF (10^{-6} M). The bound [^{3}H]PAF was separated from the free [^{3}H]PAF by immediate filtration through Whatman (Hillsboro, OR) GF/B glass-fiber filters under vacuum (Brandel system, Beckman). The reaction tubes and the filters were washed 3 times with 5 mL of precooled buffer solution. Filters were transfered to polyethylene vials filled with 10 mL liquid scintillation fluid (Instagel, Packard, Fullerton, CA). The radioactivity was measured with an LKB (Piscataway, NJ) β counter with 45% efficacy.

RESULTS

[^{3}H]PAF binding to platelet membranes. Binding of [^{3}H]PAF to rabbit platelet membranes was specific, saturable and of high affinity. A high specific binding representing 60-70% of the membrane-associated radioactivity was obtained under our experimental conditions, which included Mg^{2+} ions in the binding buffer solution.

Before interpreting any change of PAF binding in rabbit platelet membranes, the effect of storage at −80°C on the PAF binding capacity was investigated (Table 1). Some differences were noted when we compared fresh and frozen preparations; in the subsequent studies, we used frozen membranes with well-reproducible [^{3}H]PAF binding characteristics.

Comparative displacement of [^{3}H]PAF from rabbit platelet membrane with PAF, lysoPAF and PAF antagonists. [^{3}H]PAF on platelet membranes was fully displaced by PAF, L 652731, BN 52111, BN 52115, BN 52021 and WEB 2086 with IC_{50} of 2 nM, 20 nM, 35 nM, 35 nM, 200 nM and 150 nM, respectively (n=4) (Fig. 2). The maximum displacement obtained with lysoPAF at the highest concentration used (10^{-6}M) was 20%.

Regulation of PAF binding by PAF and lysoPAF. When the membranes were not washed after 20-min preincubation of platelet membranes with PAF (10^{-6} M) followed by a 90-min incubation period with [^{3}H]PAF at 0°C, no significant specific binding was observed, which suggests full occupancy of the PAF binding sites (Fig. 3). Five extensive washings of the membranes after preincubation with PAF resulted in a partial recovery (30%) of [^{3}H]PAF binding. Increasing concentrations of PAF (10^{-9} M, 10^{-8} M, 10^{-7} M) decreased the number of binding sites con-

TABLE 1

[^{3}H]PAF Binding in Fresh and Frozen Rabbit Platelet Membranes

	K_d (nM)	B_{max} (pmole/mg)
Fresh membranes (n=10)	1.96 ± 0.25	2.08 ± 0.15
Frozen membranes (5 days) (n=9)	4.73 ± 0.13	1.25 ± 0.07

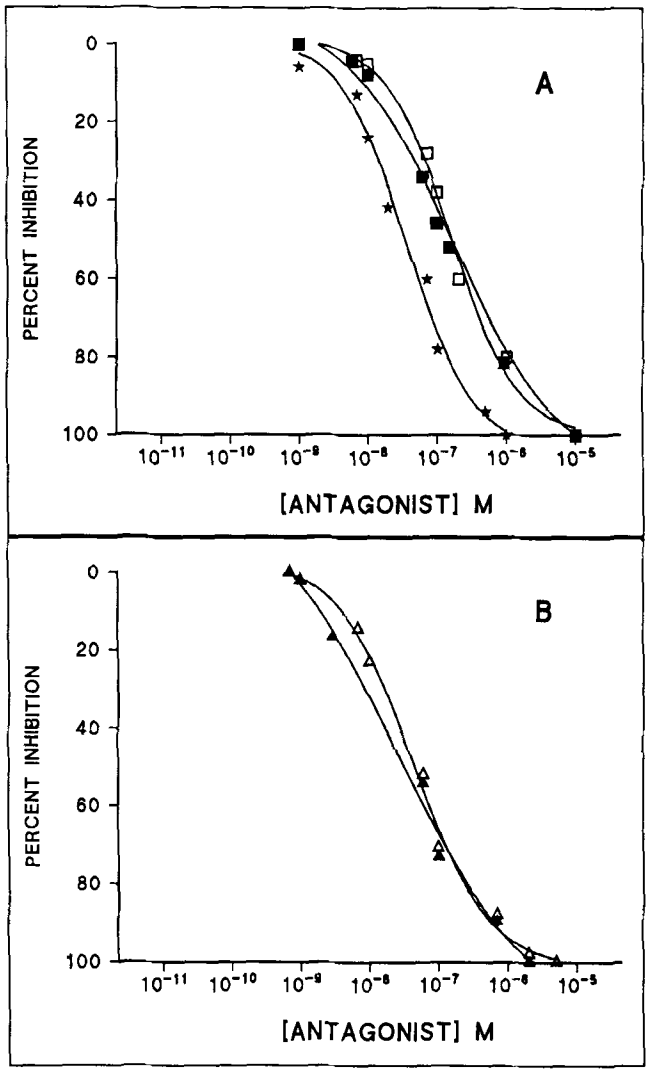

FIG. 2. A, Potencies of L 652731 (★) IC_{50} = 2 ± 0.2 10^{-8} M, BN 52021 (□) IC_{50} = 2 ± 0.1 10^{-7} M to inhibit [^{3}H]PAF specific binding in rabbit platelet membranes. B, Potencies of BN 52111 (▲) IC_{50} = 3.5 ± 0.05 10^{-8} M and BN 52115 (△) IC_{50} = 3.5 ± 0.04 10^{-8} M to inhibit [^{3}H]PAF specific binding in rabbit platelet membranes. The experiments were performed in tris 10 mM, $MgCl_2$ 50 mM, BSA 0.025% pH 7, and each point is the average of four different experiments.

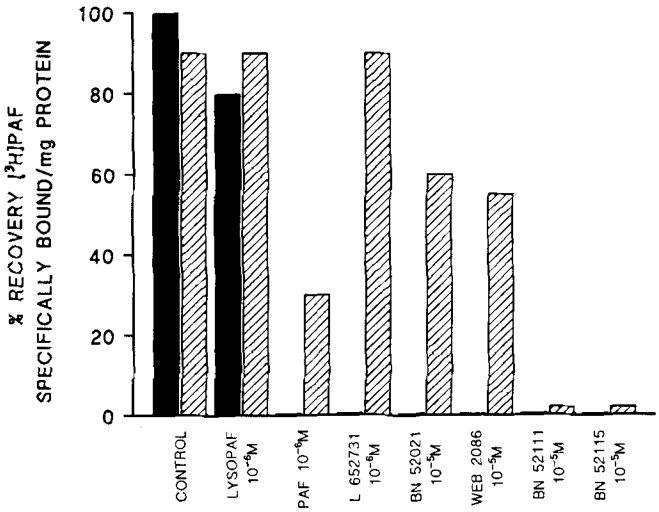

FIG. 3. Percent recovery of [^{3}H]PAF specific binding after five extensive washes (hatched bar) and without wash (solid bar) in rabbit platelet membranes preincubated in the presence of PAF, lysoPAF, L 652731, BN 52021, WEB 2086, BN 52111 and BN 52115.

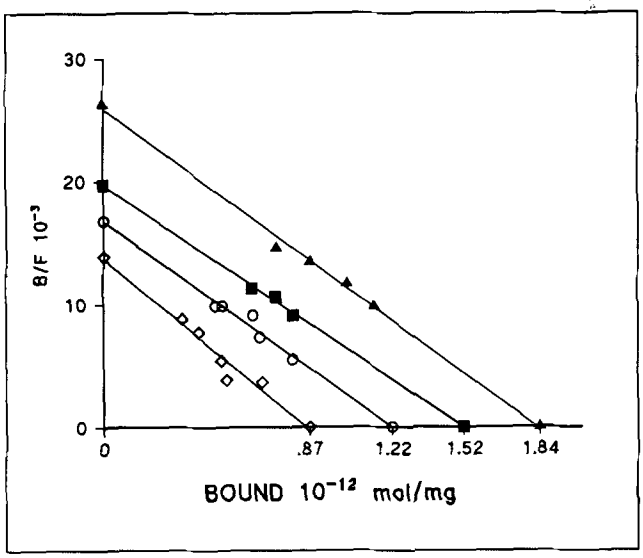

FIG. 4. Scatchard plot analysis of [^{3}H]PAF binding after preincubation with increasing concentrations of PAF:control (▲), PAF 10^{-9} M (■), 10^{-8} M (○), 10^{-7} M (□). The experiments were performed in tris 10 mM, $MgCl_2$ 5 mM, BSA 0.025%, pH 7.

centration-dependently, without significantly changing the affinity. The K_d and B_{max} values obtained are presented in Figure 4. A time-course study with 10^{-6} M PAF revealed that the effect was time-dependent; the apparent B_{max} was decreased by 50% after 15 min and reached a plateau (−70%) after 20 min.

After preincubation with lysoPAF (10^{-6} M), without washing the membranes, the [^{3}H]PAF specific binding to the membranes represented about 80% of that of the control, which suggests that only 20% of the PAF binding sites were occupied. After washing the platelet membranes, 100% of the specific binding was recovered.

Modulation of PAF binding sites by PAF antagonists. When the membranes were not washed after 20-min preincubation with L 652731, BN 52021, WEB 2086, BN 52111 or BN 52115, followed by 90-min incubation at 0°C with [^{3}H]PAF, no significant [^{3}H]PAF specific binding was obtained. Extensive washing of the membranes after preincubation resulted in full recovery of the specific binding when L 652731 was used, partial recoveries (60% and 55%) when BN 52021 and WEB 2086 were used and no recovery when BN 52111 and BN 52115 were used (Fig. 3). Scatchard plot analysis of the binding data indicated that preincubation of the membranes with L 652731 followed by extensive washings did not modify [^{3}H]PAF binding characteristics (Fig. 5A). A significant increase in K_d and decrease in B_{max} were observed for BN 52021 (Fig. 5B), and a significant decrease in K_d value was observed for WEB 2086 (Fig. 5C).

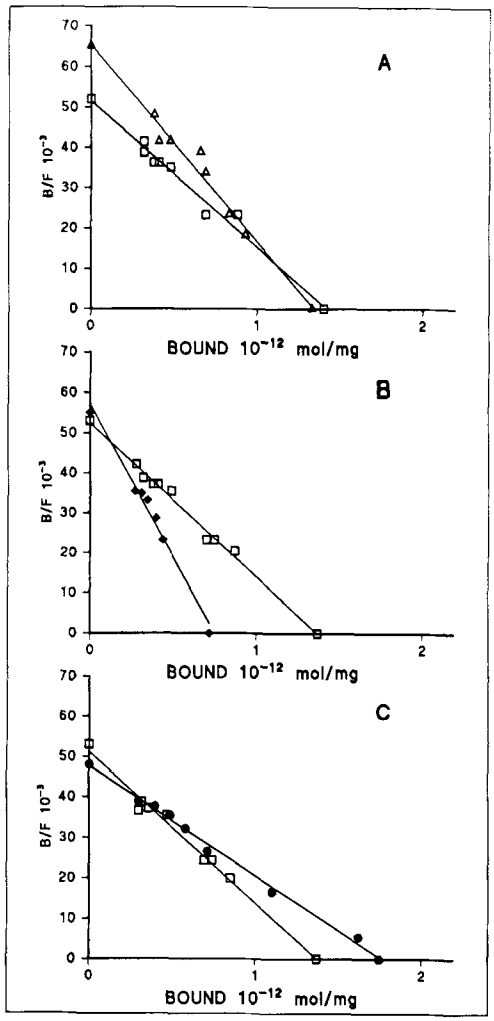

FIG. 5. Scatchard plot analysis of [^{3}H]PAF binding in rabbit platelet membranes after preincubation with A, buffer (□) and L 652731 (△); B, buffer (□) and BN 52021 (◆); C, buffer (□) and WEB 2086 (●).

DISCUSSION

The characteristics of PAF binding to rabbit platelet membranes were studied before and after 20-min preincubation at 25°C in the presence of PAF, lysoPAF or five competitive PAF antagonists. All the binding experiments were performed using frozen membrane preparations (5 days maximum), for which [^{3}H]PAF binding characteristics are well defined.

Preincubation of the membranes with increasing concentrations of PAF followed by extensive washings progressively decreased specific binding. Scatchard plot analysis of the data revealed a concentration-dependent downregulation of PAF binding sites, without significant change in the dissociation constant (K_d). This effect has previously been described in intact human platelets, for which no recovery of the binding was observed after preincubation of the cells with an excess of unlabeled PAF (25,26). In our experiments, 30% of the specific binding was recovered, even at the highest concentration of PAF (10^{-6} M). This discrepancy may be due to our experimental conditions (membrane preparation *vs.* intact cells) or to an easier dissociation of bound [^{3}H]PAF from its recognition sites. Increasing the preincubation time from 20 to 45 min still resulted in 30% recovery in PAF binding.

Preincubation of the membranes with various PAF receptor antagonists revealed profound differences in [^{3}H]-PAF binding. Preincubation with L 652731 followed by extensive washing did not affect [^{3}H]PAF binding capacity, which suggests that the interaction of this antagonist with the PAF receptor site is fully reversible (29).

Under the same conditions, except that L 652731 was replaced by BN 52021, only 60% of the specific binding was recovered. Scatchard plot analysis of the binding data showed that exposure of the membranes to BN 52021 decreased the maximum number of binding sites by 50%, with a slight increase of the affinity of [^{3}H]PAF for the receptor. When plasma membranes were incubated with WEB 2086, the 55% specific binding recovered corresponded to a decrease in the affinity of [^{3}H]PAF for the receptor and to an increase in the maximum number of sites.

When BN 52111 or BN 52115 were used no recovery was observed, suggesting that the interaction of each of these two compounds with PAF binding sites is probably irreversible.

These results may allow us to distinguish three classes of PAF-receptor antagonists—fully reversible, partially reversible and totally irreversible. The interaction of the various antagonists with the PAF receptor appears to be dependent on their chemical structure rather than on their relative affinity for this receptor. The partially reversible and irreversible compounds may bind tightly to the PAF receptor and thus are difficult to wash out; they may also change or regulate, as PAF itself does, the binding characteristics of PAF in platelet membranes. It is also conceivable that the antagonists structurally unrelated to PAF recognize different components of PAF binding.

It is of interest to note that BN 52111 and BN 52115 are structurally related to PAF and exhibit a long lasting effect *in vivo* (30). Indeed, structure-activity relationships have been established demonstrating the necessity for a long alkyl group and for a charge transfer group to express maximal antagonistic and irreversible action (22).

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The Origin of Palmitic Acid in Brain of the Developing Rat

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A rat milk substitute containing lower amounts of palmitic and oleic acid in the triacylglycerols in comparison to natural rat milk was fed to artificially reared rat pups from day 7 after birth to day 14. Pups reared by their mother served as controls. Free trideuterated (D_3) palmitic acid [$(C^2H_3)(CH_2)_{14}COOH$, 98 atom % D] and free perdeuterated (D_{31}) palmitic acid [$C_{15}{}^2H_{31}COOH$, 99 atom % D] in equal quantity were mixed into the triacylglycerols of the milk substitute in an amount equal to 100% of the palmitic acid in the triacylglycerols. A control milk substitute contained unlabeled free palmitic acid in an amount equal to 100% of the palmitic acid in the triacylglycerols of the milk substitute. The objective was to determine if palmitic acid in the diet contributed significantly to the palmitic acid content of developing brain and other organs. The methyl esters of the fatty acids were analyzed by gas chromatography and the palmitic acid methyl ester was examined by fast atom bombardment mass spectrometry. The proportion of deuterated methyl palmitate as a percentage of total palmitate was determined; 32% of the palmitic acid in liver and 12% of the palmitic acid in lung were trideuterated and perdeuterated palmitic acid in approximately equal amounts. The brain, by contrast, did not contain the deuterated palmitic acid moiety. Quantitation of palmitic acid and total fatty acids revealed a significant accumulation in organs in the interval from 7 to 14 days of age. Under our experimental conditions, labeled palmitic acid does not enter the brain. Consequently, we conclude that the developing brain produces all required palmitic acid by *de novo* synthesis.
Lipids 27, 587-592 (1992).

Any substance which enters the brain must do so through capillary endothelial cell membranes or pores within these membranes, as adjacent cells possess tight junctions which prevent the passage of high molecular weight substances (1). Lipid soluble substances are assumed to directly penetrate the membranes of the cells marking the blood brain barrier, while lipid insoluble substances such as glucose are assumed to enter *via* specialized pores, utilizing additional transport proteins which have high affinity for such substances (1). Recent evidence also suggests that the glycoprotein coated surface proteins of these cells lend immense negative charges, repelling most particles (2).

Many studies have been carried out to determine the penetration of labeled fatty acids past the blood brain barrier. These studies can be classified into three general types: labeled fatty acids were i) injected into the carotid artery (3); ii) injected into some other body site, for example, the femoral artery (4), the jugular vein (5), the peritoneal cavity (6,7) or the stomach (8); iii) administered orally (9-12). In a recent study, adult rats were fed a diet deficient in essential fatty acids and then fed free octadeuterated arachidonic acid (12). Gas chromatographic/mass spectrometric analyses of fatty acids from their organs showed that 26.6% of the arachidonic acid of brain lipids was in the octadeuterated form (12). This study indicated that deuterium labeled arachidonic acid crosses the blood brain barrier. The present study was designed to determine if the abundant saturated fatty acid, palmitic acid, could gain access to the rapidly growing brain when supplied in the diet.

MATERIALS AND METHODS

Animals. Pregnant Sprague Dawley rats (second or third litter experience) were obtained from Bantin and Kingman (Fremont, CA) at 14 days of gestation and the time of parturition carefully noted. Postnatal day 0 was the calendar day on which the rat pups were born. Litters remained undisturbed until day 5. Animals were selected and artificially reared as described previously (13). The artificially reared rats numbered seven; four were fed deuterium labeled free palmitic acid added to the milk (D-milk) and three were fed a control milk (C-milk) containing unlabeled free palmitic acid in an amount equal to the deuterated free palmitic acid in D-milk.

At 14 days of age artificially reared (AR) and maternally reared (MR) pups were anesthetized with pentobarbital (50 mg/mL) at a dosage of 50 mg/kg body weight. The thorax was dissected open and then blood was taken from the right atrium into a syringe containing 0.1 mL heparin (1000 units/mL). The animal was then perfused slowly through the left ventricle with 30 mL of 38°C phosphate buffered saline containing heparin (5 mL/L) and 1% sodium sulfate (1.0 mL/L). Brain, lung and liver were removed and weighed after the perfusion. The weight of lung tissue varied markedly according to how much saline was trapped in the organ.

Four MR rats were sacrificed by decapitation on day 7 and day 14; MR rat pups killed on day seven were not perfused, as heart tissue was too delicate to withstand perfusion. Brain, liver and lung from these animals were handled as described above.

Rat milk substitute. The basic procedure for the preparation of Rat Milk Substitute (RMS) has been described extensively (13). A protein-rich premilk, which contained minimal fat of mammalian origin, was constituted from a custom prepared casein-rich protein powder (Ross Laboratories, Columbus, OH). The milk fat was constituted from a mixture of four oils of vegetable origin in the ratio 1:1:2:8, by weight, with soy oil: MCT (medium chain triacylglycerol supplied by Mead Johnson, Evansville, IN); safflower oil: MCT (Captex no. 8323, custom prepared (13) by Capital City Products, Janesville, WI) to yield a fat blend low in palmitic and oleic acids as compared to the fat of rat milk. Vitamins, minerals and other constituents

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Abbreviations: AR, artificially reared; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; MCT, medium-chain triacylglycerol; MR, maternally reared; RMS, rat milk substitute.

were added to yield a milk substitute which closely resembled rat milk in its gross and detailed composition (13). The gross composition of the milk substitute was as follows in g/L, protein 95; carbohydrate 34; fat 122; ash 11.

The rat milk substitute, without added free palmitic acid, contained 310 mg palmitic acid/100 g fat and 524 mg oleic acid/100 g fat. This milk substitute was shown to contain a total triacylglycerol content of 10.7 g/100 g of milk, with palmitic and oleic acids comprising 2.9% and 4.9% of the total fatty acids (Ross Laboratories). An additional 310 mg of deuterium labeled free palmitic acid (Cambridge Isotope Laboratories, Woburn, MA) was added per 100 g of fat to the oil mixture during preparation of the milk substitute (D-milk). Of this 310 mg, 155 mg was trideuterated free palmitic acid (98 atom % D), with the ω methyl being fully deuterated. The other 155 mg was perdeuterated free palmitic acid (99 atom% D). Unlabeled free palmitic acid (310 mg/100 g fat), 99% pure (Sigma Chemical Company, St. Louis, MO), was added to the control milk (C-milk). Other than containing labeled or unlabeled free palmitic acid, the C-milk and D-milk substitutes were identical in composition.

Preparation of fatty acid methyl esters. Organs from 7- and 14-day-old MR and AR rats were saponified overnight in one volume of 30% KOH (w/vol) at 70°C in glass tubes which were sealed with Teflon-lined caps. Fatty acids were extracted as described previously (14) and their methyl esters prepared (15). The derivatized fatty acids were then taken to dryness under N_2 gas and dissolved in 600 μL hexane for gas chromatography or 500 μL methanol for high-performance liquid chromatography (HPLC).

HPLC. Samples of 100–250 μg methyl esters of fatty acids in methanol from each of the organs were applied to an HPLC column equilibrated with HPLC grade, methanol/H_2O (95:5, vol/vol). The column, AXXI-CHROM C-18 (ODS), 5 μm, 4.6 mm i.d., 250 mm long (Cole Scientific Inc., Calabasas, CA) was fitted with a compatible RP-18 "Newguard" precolumn, 3.2 mm × 15 mm (Brownless Labs Inc., Santa Clara, CA) and was operated at 34°C. The HPLC system consisted of the model 110A Altex pump, Rheodyne injection valve with 100 μL injection loop, Hitachi model 100-30 spectrophotometer, Shimadzu model C-RIA Chromatopak and Eldex column temperature control unit (Cole Scientific Inc.). Esters of fatty acids were eluted from the column with methanol/H_2O (95:5, vol/vol) at a flow rate of 0.7 mL per minute and detected at 220 nm. Fractions were collected in solvent rinsed glass vials on an LKB model 2112 Redirac fraction collector (LKB-Produkter, Bromma, Sweden) set at 3 min per vial. The fraction containing palmitate and oleate was collected as a single peak in a single vial, retention time 18 min, and care was taken to ensure that the entire peak was included. These fractions were dried and transferred in hexane to 1 mL borosilicate conical vials ready for analysis by mass spectrometry and capillary gas chromatography to check for the presence of perdeuterated methyl palmitate.

Fast atom bombardment mass spectrometry. A double focusing sector field mass spectrometer (Model MS-9) made by Associated Electrical Industries (Manchester, U.K.) was used for the analysis of all samples. A custom-built FAB gun was retrofitted into the source chamber of this mass spectrometer (Chemical Instrumentation Division, Chemistry Department, UCLA, Los Angeles, CA). In all the analyses, a xenon beam of 5 kvolts energy was used. The FAB target consisted of a stainless steel tip of 2.5 mm diameter. Mass spectral ion signals were processed in real time by a computerized data system interfaced with the mass spectrometer. About 2 microliters of *m*-nitrobenzyl alcohol coated on the tip served as a matrix. The sample to be analyzed was dissolved in hexane. One microliter of this solution was placed onto the probe tip that was already coated with *m*-nitrobenzyl alcohol. Good protonated parent ion signals were detected with samples as low as 2 to 5 picomoles. In experiments to assess limits of detection of labeled palmitic acid in the presence of unlabeled palmitic acid, a range of mixtures of D_0 palmitic acid with D_3 palmitic acid, and D_0 palmitic acid with D_{31} palmitic acid were examined. Trideuterated and perdeuterated palmitic acid were observed at the lowest proportion tested; at 0.02% of D_0 palmitic acid (data not shown).

Absolute ion peak intensity and percent base peak intensity of the protonated parent ion $[M + 1]^+$ were used to quantitate the mixture of trideuterated, perdeuterated and unlabeled palmitates. Standards containing variable quantities of each palmitate were also analyzed to ensure a proper determination of the percentages of each type of palmitate. These mixtures were derivatized and collected by HPLC in a manner identical for the purification of samples from organs.

Gas chromatography. Known aliquots of fatty acids from each tissue were dried prior to methanolysis and a precisely known amount of an internal standard of heptadecanoic acid methyl ester (17:0, 99% pure, AllTech Associates Inc., Deerfield, IL) was added. Amounts added were estimated to be equal to the palmitate content of the sample. Methanolysis then proceeded as previously described for the preparation of samples for HPLC. Standards of fatty acid methyl esters were obtained from Supelco Inc. (Bellefonte, PA) and included palmitate, oleate and linoleate.

The capillary column used was 10% carbowax, 0.5 μm film thickness, 30 m long with an internal diameter of 0.32 mm (Cole Scientific Inc.). A Shimadzu Gas Chromatograph model GC-14A connected to an Axxiom Chromatography Data-System model 727 was used. Helium was used as the carrier gas at 0.6 kg/cm^2 column pressure. Injector temperature was 175°C, FID 200°C, initial oven temperature 140°C for 0.5 min isothermally before the temperature program was introduced. A temperature rate increase of 4°C/min to 190°C and isothermal conditions at 190°C for 5 min were followed by a second temperature rate increase of 3°C/min from 190°C to a final temperature of 223°C. This temperature was maintained isothermally for 15 or 30 min to complete the program, depending upon which tissue samples were analyzed. Profiles for fatty acid methyl esters for brain and liver were obtained in 54 min and for lung in 39 min.

Statistics. Chromatographic analyses of the methyl esters of fatty acids from organs were done a minimum of two times and area percent of peaks and concentrations were calculated for each using the internal standard. Mean and standard error were calculated for the data from brain and liver for palmitate, oleate and linoleate. The percentages of total fatty acids contributed by palmitate, oleate and linoleate were calculated.

Differences between animal groups were analyzed by a

nested mixed model program (16) for analysis of variance, where group (AR and MR) and individual animal contributions to variances were analyzed. This design treated the two groups (AR and MR) as fixed while animals were nested within the groups and treated as random.

Because many comparisons between means were made, a cut off value for significance was set at $P < 0.02$, to decrease the chance of random acceptance of the null hypothesis in error which is possible, with an alpha level of 0.05. All other statistical comparisons were with Student's *t*-test for the difference between means with a significance level of $\alpha = 0.05$.

RESULTS

Weight gain. Animals reared by their mothers or by the artificial rearing system showed no significant differences between the means for weight gain. MR animals were weighed on days 6, 8, 11 and 13. The confidence intervals for AR animals on these days *vs.* those for MR are overlapping and not significantly different by the Student's *t*-test ($\alpha = 0.05$) (data not shown).

Distribution of deuterium labeled palmitic acid. Table 1 shows results which were obtained by fast atom bombardment mass spectrometry for palmitate from organs. Palmitic acid from animals receiving C-milk contained only the nondeuterated form of palmitate, as confirmed by the FAB mass spectrum showing a base peak at *m/z* 271 that corresponds to the protonated molecular ion $[M + 1]^+$ of the methyl ester of nondeuterated palmitate. The mass spectra of palmitate from animals receiving D-milk showed the presence of nondeuterated palmitate as base ion at *m/z* 271 for all samples. Palmitate from liver and lung, however, contained trideuterated (*m/z* 274) and perdeuterated (*m/z* 302) palmitates. The amount of each palmitate as a percentage of total was calculated from their contribution as percent base intensity. Their percentages in liver were 17.5 ± 2.4 and 15.2 ± 2.3 (mean ± SD) of total palmitate, for trideuterated and perdeuterated palmitate, respectively. In lung they amounted to 7.4 ± 1.1% and 5.2 ± 0.5% of total palmitate, respectively. Palmitic acid from brain, by contrast, contained no perdeuterated palmitic acid and showed only a trace of the molecular ion representing trideuterated palmitic acid. The presence of an ion, *m/z* at 274, that is, $[M + 1]^+$, the base ion for trideuterated palmitate, in brain palmitic acid may account for 0.48 ± 0.21% of the total palmitate. However, the ion at M + 1 for trideuterated palmitate can contain a contribution from nondeuterated palmitate (*m/z* at 271, $[M + 1]^+$) at M + 4, an isotopomer of nondeuterated palmitate. This small contribution is confirmed by a comparison with the spectrum of palmitate from brain of control animals (see Table 1, columns 5 and 6).

Palmitate from the D-milk was analyzed by fast atom bombardment-mass spectrometry and the results are presented in Table 2. The amount of deuterated free palmitic acid added to D-milk was based on the palmitic acid content of the triacylglycerol in a milk substitute prepared earlier for analyses by Ross Laboratories. The rat milk substitute for this study was formulated to contain the same nutrient composition. The data presented in Table 2, showing a greater proportion of palmitic acid as deuterated palmitic acid than calculated in the milk substitute, indicate that the amount of natural palmitic acid was lower in the milk prepared for this experiment.

Palmitic acid and total fatty acids in brain and liver. Tables 3 and 4 show the amounts of palmitic acids, total fatty acids and deuterated palmitic acids in brain and liver. There were no significant differences between the groups for palmitic acid and total fatty acids in brain and liver except for an increased amount of total fatty acids in the liver of the AR animals (*P* value <0.03). This difference arose because the livers from AR animals were heavier than livers from control animals (data not shown).

Table 4 shows the mass of deuterium labeled palmitic acid incorporated into brain and liver. Palmitic, oleic and

TABLE 1

Analysis of Palmitate by FAB-MS[a]

		Percent base intensity for[b]					
		D_0 palmitate[c]		D_3 palmitate		D_{31} palmitate	
Organ	Ion	D-Milk	C-Milk	D-Milk	C-Milk	D-Milk	C-Milk
Brain	M + 2	18.98 (1.2)	18.17 (0.8)	0.00	0.00	0.00	0.00
	M + 1	100.00 (0.0)	100.00 (0.0)	0.48 (0.2)	0.20 (0.2)	0.00	0.00
	M	3.47 (0.6)	2.90 (0.5)	2.10 (0.5)	0.40 (0.4)	0.00	0.00
Liver	M + 2	26.18 (2.5)	18.53 (1.1)	3.60 (0.6)	0.00	3.05 (0.8)	0.00
	M + 1	100.00 (0.0)	100.00 (0.0)	17.53 (2.4)	0.00	15.18 (2.3)	0.00
	M	4.68 (1.0)	3.20 (1.4)	3.55 (0.3)	1.80 (0.4)	3.37 (0.5)	0.00
Lung	M + 2	21.18 (2.6)	19.80 (0.7)	0.73 (0.2)	0.10 (0.1)	0.33 (0.1)	0.00
	M + 1	100.00 (0.0)	100.00 (0.0)	7.37 (1.1)	0.70 (0.7)	5.20 (0.5)	0.00
	M	1.20 (0.6)	2.70 (0.9)	2.90 (1.6)	3.35 (0.6)	0.67 (0.1)	0.00

[a] Animals were fed either D-milk or C-milk and the total fatty acids were obtained from brain, liver and lung. The methyl esters of fatty acids were prepared and purified by HPLC and the palmitate fractions examined by fast atom bombardment mass spectrometry (see Materials and Methods). Data are presented as the mean ± SD. Four animals were fed D-milk and three animals were fed C-milk.

[b] Percent base intensity is obtained from the absolute intensity for a particular ion or ion fragment, expressed as a percentage of the absolute intensity of the ion identified as the base peak.

[c] D_0, D_3 and D_{31} are natural, trideuterated and perdeuterated palmitic acids, respectively. The mass (M) of their methyl esters is 270, 273 and 301, respectively.

TABLE 2

Analyses of Palmitates from D-Milk by FAB-MS[a]

Palmitate	Base intensity (%)[b]			Absolute[c] intensity	Amount of palmitate present as % of total	
	M	M + 1[d]	M + 2	M + 1	By analysis[e]	By weighing[f]
D_0[g]	4.3	100	31.5	2039	42.5	50.3
D_3	6.7	71.9	12.9	1446	30.5	24.7
D_{31}	7.6	64.0	4.8	1304	27.1	25.0

[a] Palmitic acid was isolated from a sample of the milk substitute, purified and prepared for analyses as described in Materials and Methods. The values are averages from quadruplicate analyses.

[b] See Table 1.

[c] The absolute intensity of the base peak $[M + 1]^+$ for each palmitate in the sample, natural, trideuterated and perdeuterated palmitate.

[d] The base peak in the mass spectra of palmitate is at $[M + 1]^+$, because the molecular ion is protonated.

[e] The absolute intensity for $[M + 1]^+$ for each form of palmitate was summed and the contribution by each expressed as a percentage; the values for the amount of each palmitic acid in the milk is the average of the duplicate analyses from one milk sample.

[f] The amounts added by weighing, corrected for purity, as indicated by the supplier, were summed and the contribution by each expressed as a percentage. The mass of natural palmitate already present in the milk was calculated from an analysis provided by Ross Laboratories (Columbus, Ohio).

[g] D_0, D_3 and D_{31} are natural, trideuterated and perdeuterated palmitate, respectively.

TABLE 3

Palmitate and Total Fatty Acids in Brain and Liver[a]

	Palmitate			Total fatty acids		
Group[b]	MR	MR	AR	MR	MR	AR
Age in days	7	14	14	7	14	14
(n)	4	4	7	4	4	7
Brain mg/brain	2.5 ± 0.1	5.6 ± 0.4	5.7 ± 0.5	6.4 ± 0.3	18.6 ± 0.1	18.2 ± 0.6
Liver mg/liver	2.8 ± 0.3	5.5 ± 0.1	5.1 ± 0.7	9.0 ± 0.2	20.2 ± 0.3	26.4 ± 0.6

[a] Fatty acids were extracted from organs of rat pups in the MR group who served as controls and in the AR groups who were fed either unlabeled palmitic acid or the deuterated palmitic acids in the milk substitutes as described in Materials and Methods.

[b] MR groups are the mother reared controls, AR groups are the artificially reared groups. The fatty acids were separated by capillary gas chromatography as described in Materials and Methods. Values are mg per organ and expressed as the mean with standard deviation; (n) shows the number of animals in each group.

TABLE 4

The Amount of Natural and Deuterated Palmitic Acid in Liver and Brain of Animals Fed D-Milk[a]

Palmitate	D_0[b]	D_3	D_{31}
		mg/g wet weight	
Liver	4.00 ± 0.42	0.69 ± 0.13	0.59 ± 0.11
Brain	4.94 ± 0.42	0.03 ± 0.01	0.00

[a] The data are derived from liver and brain of animals fed deuterated palmitic acid (Table 3). Palmitic acid was isolated from organs, purified and prepared for analyses by FAB-MS and capillary gas chromatography as described in Materials and Methods. Values are means with standard deviation and are calculated from the results of the analyses by capillary gas chromatography and the proportion of each as determined by analyses by FAB-MS (Materials and Methods, Tables 1 and 2).

[b] D_0, D_3 and D_{31} are natural, trideuterated and perdeuterated palmitate, respectively.

linoleic acids, as a percent of total fatty acids in rat milk, the rat milk substitute and the brain, liver and lung of MR and AR rats are shown in Table 5. Although the amounts of palmitic and oleic acids in the fat of the milk substitute were reduced by about 88% and 72%, respectively, while linoleic acid was increased by about 225% from the amounts in rat milk, there was no difference in the concentration of these fatty acids in the brains of AR and MR rats. A similar statement cannot be made for the liver and lung of these animals, because, with the exception of oleic acid in lung, the amounts of each of these fatty acids in liver and lung of AR rats were significantly different from the amounts in liver and lung of MR rats (Table 5).

DISCUSSION

There were design aspects to our study which bear highlighting. Our approach takes into consideration the

TABLE 5

Fatty Acids in Milk and Organs[a]

	Milk		Organs					
			Brain		Liver		Lung	
Group	Rat milk[b]	RMS[c]	AR	MR	AR	MR	AR	MR
Fatty acids	Percentage of total fatty acids							
Palmitate	18.4-32.6	2.9	27.9 ± 0.2	26.4 ± 0.4	17.2 ± 0.5[d]	23.6 ± 0.4[d]	32.3 ± 0.7[e]	35.1 ± 0.2[e]
Oleate	15.2-20.4	4.9	12.9 ± 0.2	11.2 ± 0.1	7.1 ± 0.1[f]	5.8 ± 0.2[f]	17.6 ± 0.6	19.8 ± 0.2
Linoleate	6.0-11.4	19.6	1.7 ± 0.1	1.3 ± 0.1	27.6 ± 0.6[g]	16.3 ± 0.3[g]	20.0 ± 0.5[h]	15.3 ± 0.2[h]

[a]Fatty acids were isolated from the rat milk substitute and organs, purified and prepared for analyses by capillary gas chromatography as described in Materials and Methods. The data are from animals whose organs were analyzed for their fatty acid content as shown in Tables 1 and 3. Superscripts d-h, for liver and lung, values with the same superscript letter for a particular fatty acid are significantly different, $P < 0.02$, see section on statistics (Materials and Methods).

[b]Percentages taken from ref. 13.

[c]Percentages taken from the analysis of the rat milk substitute by Ross Laboratories (Columbus, OH). The number of animals in each group is given in Table 3. Values are mean ± SEM.

concern expressed by Pardridge and Mietus (3) that lipids such as cholesterol or palmitate should be presented to the blood brain barrier as a component of normal circulating lipids at this interface, otherwise the outcome may be artifactual. In our study, deuterated free palmitic acid from the milk was subjected to normal digestion, absorption and intestinal processing and transport, and was presented within the natural milieu of the various circulating lipoproteins to the cellular interface of organs such as liver, lung and brain. It was shown by studies on humans that deuterium labeled fatty acids were freely absorbed and transported (17-20). The seven-day exposure of the organs to deuterated palmitic acid, in our study, was long enough to allow for extensive equilibration of deuterated palmitic acid into accessible compartments. It was considered important to perfuse the animals to decrease the possibility of contamination from label carried by blood and trapped within organs before organs were removed. There is the possibility however, that label within the cells of the capillary network permeating the brain and other organs is a source of contamination; the capillary endothelial cellular network was included with brain when brain lipids were extracted, yet it is considered to be extra cerebral tissue. A final highlight is that the mass spectrometry technique of fast atom bombardment finds selectively and unambiguously the stable isotopic form of the substance which is fed and is to be quantitated.

Blood brain barrier. Mead and associates (4,10,21) performed many experiments to determine if brain incorporated palmitic acid directly. Their conclusion that labeled palmitic acid, injected intraperitoneally or administered orally, became incorporated into brain lipids was developed from analysis of the percent of radioactivity present in the carboxyl carbon, *vs.* other carbons within the fatty acyl chain of brain palmitic acid. Since the palmitic acid in brain acquired and retained a higher carboxyl radioactivity, it seemed that much of it was probably incorporated unchanged (4,21). Theoretically, if palmitic acid was formed in brain by *de novo* synthesis there would be a random distribution of radioactivity within the carbon chain from uptake of radioactive acetate transported to brain after β-oxidation of radioactive palmitic acid in extra neural organs such as the liver. Earlier, it was observed that there was a relative carboxyl activity of 20.7% for the carboxyl carbon of palmitic acid in brain from weanling rats after the injection of labeled acetate (10). Since palmitic acid is made up of eight "acetyl-units," the expected relative carboxyl activity should be 12.5%. This difference suggests that the carboxyl terminus of palmitic acid, when synthesized from radioactive acetate, exchanges more rapidly than the other labeled two carbon units within the hydrocarbon chain. Thus it is not feasible to distinguish with certainty, the proportion of the palmitic acid which is unchanged [1-^{14}C]palmitic acid as fed and palmitic acid which has been preferentially labeled on the carboxy-terminus through an exchange with the radioactive acetate pool.

Other investigators studying uptake of radioactive palmitic acid into brain have found varying levels of oxidation products. In a study in which the oxidation of [1-^{14}C]palmitic acid and other metabolites was compared in various brain areas, little incorporation of label into amino acids associated with the Krebs cycle was observed (6). Another study using filtered whole blood containing albumin bound [U-^{14}C]palmitic acid showed a quantitative oxidation of palmitic acid to $^{14}CO_2$ of only 2.8% (22). It was suggested this small amount of oxidation could be occurring in extra cerebral tissues (22). A brief survey of the relevant literature revealed many investigations which showed that varying amounts of radioactively labeled palmitic acid can access brain (3,4,6,8,10,21).

The only previous study in which a deuterium labeled saturated fatty acid was used in a study of transport across the blood brain barrier was done more than fifty years ago (9). In this study the unsaturated fatty acids in linseed oil were hydrogenated with deuterium. The treatment yields a fat containing about 90 percent deuterated stearic acid with either 2, 4 or 6 deuterium atoms from the oleic, linoleic and linolenic acids respectively. The fat was fed to adult male rats by stomach tube; then brain, liver, intestine and the remaining carcasses were examined for their content of deuterium labeled fatty acids. A very low uptake of deuterium labeled fatty acid into brain and a large deposition into liver and other organs were observed (9). Waelsch and associates (9,23,24) concluded that

"the brain synthesizes the fatty acids it needs, wholly or in large part, and does not depend on an external source of supply."

Perdeuterated and deuterated substances are known to have access to the brain (25–29) and have been used to study the half lives of rapidly metabolized drugs, because they are not degraded as quickly as the unlabeled forms (26–29). During the seven-day period of our study, a large net increase in fatty acids and palmitic acid occurred in the liver and to a greater extent in brain. In addition, the effect of an altered fatty acid composition in the fat of the milk substitute as compared to maternal milk, had a profound influence on the content of palmitic, oleic and linoleic acids in liver and in lung. By contrast, the concentration of these fatty acids was not altered in the brain.

Our study shows under the experimental conditions which prevailed an absolute exclusion of perdeuterated palmitic acid from brain tissue. Although it is possible that a very small amount of trideuterated palmitic acid may have been trapped in the brain capillary endothelial cell network, it is clear from our study that trideuterated palmitic acid does not access the brain. By contrast, octadeuterated arachidonic acid does access the adult brain to accumulate in significant amounts (12). It is logical to suggest that unsaturated fatty acids which can be produced from palmitic and stearic acid may also be biosynthesized in brain to meet its needs, consequently, we speculate that a selective transport system across the blood brain barrier may exist for the essential fatty acids. We conclude that the brain synthesizes *de novo* all the palmitic acid it requires.

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Arachidonic Acid Biosynthesis in Non-Stimulated and Adrenocorticotropin-Stimulated Sertoli and Leydig Cells

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The biosynthesis of arachidonic acid in Sertoli and Leydig cells isolated from the testes of mature rats has been investigated. Both types of cells incorporated [2-^{14}C]eicosatrienoic acid from the incubation medium and transformed it into arachidonic acid. The administration of adrenocorticotropin (ACTH) to the rats decreased the Δ5 desaturating activity in the isolated testicular cells, while ACTH produced no changes in the uptake of the substrate. Similar results were obtained when ACTH was added to the incubation medium of cells isolated from non-hormone treated rats. The total fatty acid composition of the Sertoli cells isolated from ACTH-treated rats showed a significant increase in the relative percentage of 18:2n-6 and a decrease in the C_{20} and C_{22} polyenes. This may indicate that ACTH exerts an inhibitory effect on Δ6, Δ5 and Δ4 desaturase activities. Addition of corticosterone to the incubation medium also produced a significant decrease in arachidonic acid biosynthesis. Because ACTH is known to stimulate the release of corticosterone *in vivo*, both hormones may act cumulatively in the regulation of arachidonic acid metabolism in Sertoli and Leydig cells.
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Polyenoic acids derived from linoleic or α-linolenic acids are important components of testicular tissue. Rat testes contain relatively large amounts of polyunsaturated fatty acids (PUFA), mainly arachidonic and docosa-4,7,10,13,16-pentaenoic acids (1–3). The distribution of these fatty acids among the different cell types of the testes is not uniform. Thus, rat spermatids contain much more docosa-4,7,10,13,16-pentaenoic acid (22:5n-6) than do spermatocytes (4). Sertoli cells contain much lower levels of 22:5n-6 (5), and Leydig cells contain only very small amounts (6).

The pathways of polyunsaturated fatty acid biosynthesis have been studied rather extensively in whole testes and in mixtures of various testicular cells. These studies have generally shown that the main route of polyenoic fatty acid biosynthesis follows an alternating sequence of desaturation and elongation reactions, the former of which are thought to be catalyzed by fatty acid desaturases (7–9). The conversion of linoleate to arachidonate by rat Sertoli cells in primary cultures was more recently reported by Coniglio and Sharp (10). Studies carried out with subcellular fractions of whole testes demonstrated the presence of a microsomal Δ6 desaturase that converts linoleic acid to γ-linoleic acid, and a microsomal Δ5 desaturase which synthesizes arachidonic acid (20:4n-6) from eicosa-8,11,14-trienoic acid (20:3n-6) (11,12).

There is substantial evidence that fatty acid composition plays an important role in the normal differentiation of germinal cells. During sexual maturation, the testes accumulate large quantities of 22-carbon polyenes (13,14). Their levels decrease significantly when spermatogenesis is impaired (14). Moreover, in adult rats polyunsaturated fatty acid biosynthesis is markedly affected by nutritional and hormonal deficiencies (15,16).

The control of testicular function is a multihormonal process that requires the functional integrity of the hypophysis and eventual hormonal stimulation of the seminiferous tubules by Leydig cell-derived testosterone. Hypophysectomy is followed by a regression of the germinal epithelium (17) with a concomitant decrease in acyl-CoA synthetase activity (18). The administration to rats of the hypophyseal hormone adrenocorticotropin (ACTH), whose primary target organ is the adrenal gland, depresses acyl-CoA synthetase activity in adrenal and in adipose tissues (19,20), and inhibits PUFA biosynthesis in liver and in isolated adrenocortical cells (21). ACTH-secreting tumors, chronic stress accompanied by ACTH hypersecretion and prolonged administration of ACTH have been shown to produce a decrease in serum testosterone levels (22–25). The presence of pro-opiomelanocortin-derived peptides, particularly ACTH, has been demonstrated in testicular tissues. These hypophysis-derived peptides are probably responsible for the increases in cyclic adenosine monophosphate (cAMP) observed in Sertoli (26) and Leydig cells (27).

The steroidogenic capacity of Leydig cells is essential for sustaining spermatogenesis; however, this function can be modulated by Sertoli cell-secreted factors (28). Sertoli cells are also involved in the maintenance and nutrition of the germinal cells (29).

Considering that polyunsaturated fatty acid biosynthesis has been mainly studied in whole testes, that individual testicular cells are highly specialized, and that each type is essential for completion of spermatogenesis, the experiments reported here have two major objectives: (i) To demonstrate the ability of isolated Sertoli and Leydig cells to synthesize arachidonic acid; and (ii) to investigate the participation of the pituitary-adrenal axis in the biosynthesis of arachidonic acid in the isolated cells.

MATERIALS AND METHODS

[2-^{14}C]Eicosa-8,11,14-trienoic acid (47.7 mCi/mmol, 98% radiochemically pure) was purchased from Amersham International (Amersham, U.K.). Unlabeled eicosa-8,11,14-trienoic acid was supplied by Nu-Chek Prep (Elysian, MN). Bovine serum albumin (essentially free of fatty acids), trypsin, DNAse type I and diBucAMP were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ). ACTH was from Elea Laboratories

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Abbreviations: ACTH, adrenocorticotropin; cAMP, cyclic adenosine monophosphate; diBucAMP, dibutyryl cAMP; DNAse, deoxyribonuclease; KRBG, Krebs Ringer bicarbonate glucose; PUFA, polyunsaturated fatty acids.

(Buenos Aires, Argentina). All other chemicals used were of analytical grade.

Animals and experimental design. The adult male Wistar rats used (300–350 g body weight) were maintained in a temperature-controlled environment at 24 ± 1°C and subjected to a daily 12-h light/12-h dark cycle, with midnight being the midpoint in the dark period. The animals were housed in cages with free access to water and chow pellets (Cargill, type C). The rats were fasted for 24 h, then refed with the commercial diet for 2 h, and killed 12 h after the refeeding period.

Three series of experiments were performed using isolated Sertoli and Leydig cells. First, the ability of cells to incorporate eicosa-8,11,14-trienoic acid from the medium into cellular lipids and to transform it to arachidonic acid was examined with cells from four control (*i.e.*, non-hormone treated) rats. Second, the same parameters were measured in isolated cells after the administration of a single dose of ACTH to four rats. The ACTH was injected intraperitoneally at a dose of 4.5 IU per rat 30 min before sacrifice. The control group (also four rats) received saline solution instead of the hormone at the same time point. Third, we studied the effect of ACTH (250 μIU/per flask), diBucAMP (0.5 mM) and corticosterone (0.8 μM), which were added to the incubation medium, on 20:3n-6 uptake and its conversion to 20:4n-6 in cells isolated from four untreated rats. Corticosterone was initially dissolved in ethanol and then added to the incubation medium; the final ethanol concentration was 0.01% (vol/vol). The same concentration of ethanol was present in the medium of control cells.

Isolation of testicular cells. Rats were killed by decapitation with minimal handling. Testes were immediately removed, decapsulated and weighed.

Separation of dispersed Leydig cells. Leydig cells were isolated after mechanical shaking with collagenase in 25-mL flasks (Falcon, Division of Beckton Dickinson Co., Oxnard, CA) according to the procedure of Suescun *et al.* (30). Decapsulated testes were treated with collagenase (0.3 mg/mL) at a ratio of 1 mg/g of tissue using a metabolic incubator at 34°C for 10 min with gentle, constant shaking. The incubation was stopped by dilution with cold medium. The supernatant was filtered through a double layer of nylon mesh and centrifuged at 800 × *g* for 10 min, after which the pellet was resuspended in 5 mL of medium. Cell viability, as judged by trypan blue exclusion (31), was 85–90%. After counting the cells in a hemocytometer, cell concentrations was adjusted to 10^6cell/mL. The entire procedure for the isolation of Leydig cells took 50 min.

To perform incubations in a medium as simple as possible, Medium 199, the original medium described by Suescun *et al.* (30) was replaced with Krebs Ringer bicarbonate glucose (KRBG 0.1%), albumin (0.1%), pH 7.4, in all experiments.

Preparation of Sertoli cells. The remaining collagenase-treated testicular tissue was chopped at right angles and used for isolating Sertoli cell aggregates following treatment with trypsin and collagenase, as described by Dorrington *et al.* (32). The testis fragments were treated with a solution containing (per g of testis) 25 mL of 0.25% trypsin in KRBG-1% DNAse medium (Buffer A). After 20 min of incubation at 34°C in a metabolic shaker (80 oscillations/min), the dispersed material was passed through a double layer of nylon mesh to remove undigested testicular remnants. The filtrate was centrifuged at 2000 × *g* for 5 min, and the supernatant was discarded. In order to inhibit trypsin activity, the sediment was washed twice with 25 mL of Swim's S-77 medium with 10% calf serum and twice with the same volume of Buffer A. The sediment was then resuspended in 20 mL of 0.05% collagenase (prepared in Buffer A) per g of initial tissue weight and incubated at 34°C for 20 min, followed by low-speed centrifugation (2000 × *g*) for 5 min. The pellet was washed twice with KRBG, and the Sertoli cell aggregates finally obtained were resuspended in 5 mL of KRBG. The entire procedure for the preparation of Sertoli cells took approximately one hour. Sertoli cells were examined for viability (85–90%), counted as described for the Leydig cells, and immediately used. The recovery of Leydig and Sertoli cells was about 5–7 × 10^6 cells per testis.

Cytologic studies. A drop of approximately 2 μL from a pellet of either Sertoli or Leydig cell preparations was placed on slides. The drop was spread over the slide, allowed to dry until the visible liquid evaporated, and then immediately fixed in Bouins for 1 h. The smears were stained with hematoxylin-eosin, dehydrated and mounted.

Cells were counted under a light microscope, using a grid of 8 × 8 mm that was placed in the eye-piece, at a magnification of 100 times. The results showed that the Leydig cell preparation consisted of 79% Leydig cells and of 21% spermatids, spermatocytes and small cytoplasmic fragments. The Sertoli cell preparation consisted of 80% Sertoli cells and 20% spermatocytes and small cytoplasmic fragments.

Incubation procedure. Aliquots of isolated cell suspensions (10^6 cells per tube) were incubated in a final volume of 3 mL of KRBG in siliconized glassware in a metabolic shaker (80 oscillations/min) at 34°C under an atmosphere of 95% O_2/5% CO_2 for various time periods. In the first series of experiments, the cells were incubated for 3 h with a mixture of 10 nmoles of [2-^{14}C]eicosa-8,11,14-trienoic acid and increasing amounts of unlabeled eicosa-8,11,14-trienoic acid up to a concentration of 40 μM, or with eicosa-8,11,14-trienoic acid at a concentration of 15 μM (10 nmoles of the labeled acid and 35 nmoles of the unlabeled acid) for 30 to 180 min. In all experiments the acid was added as its sodium salt bound to defatted albumin according to Spector *et al.* (33).

In the second series of experiments, both types of cells were incubated for 180 min in the presence of the substrate at a concentration of 15 μM. In the third series of experiments, Leydig cells, as well as aggregated Sertoli cells, were preincubated in the presence or absence of either ACTH, dibutyryl cAMP (diBucAMP), corticosterone or the alcoholic vehicle for 30 min. At the end of this period, 15 μM of eicosa-8,11,14-trienoic acid was added to the medium and the cells were incubated for 3 h under the conditions established in the first series of assays.

Analytical methods. At the end of the incubation periods, suspended cells were collected into cold tubes and rapidly centrifuged. The cell pellets were washed twice with cold KRBG solution and resuspended in a final volume of 5 mL. An aliquot of the suspension was used to determine the viability of the cells and the amount of cellular protein (34). The viability remained constant up to the end of the incubation period. The rest of the cell suspension was centrifuged at 2000 × *g* for 5 min. The cells were saponified at 85°C with 2 mL 10% KOH in

ethanol plus 0.5 mL methanol for 45 min. After acidification of the solution, the fatty acids were extracted with light petroleum hydrocarbon (b.p. 30–40°C) and esterified with methanolic 3N HCl for 3 h at 68°C. An aliquot of the medium (1.5 mL) was treated identically to obtain the fatty acid methyl esters.

The radioactivity of the recovered methyl esters was determined in a Beckman liquid scintillation counter (Model L5-3133 P, Fullerton, CA) with 96.7% efficiency for ^{14}C. The distribution of radioactivity among the fatty acids was determined in a gas-liquid radiochromatograph equipped with a Panax proportional counter and a Suroscribe 2S recorder (Panax Equipment Ltd., Redhill, Surrey, England). The column was packed with 10% SP-2330 coated on Chromosorb WAW-DMCS (100–200 mesh; Supelco, Bellefonte, PA). Percentage conversion was calculated from the distribution of radioactivity between substrate and products measured directly on the radiochromatogram. The composition of fatty acid methyl esters was determined by gas-liquid chromatography using a Hewlett-Packard chromatograph (model 5840 A, Palo Alto, CA) equipped with a flame ionization detector. The column was packed as described for radiochromatography. The oven temperature was programmed from 140 to 220°C at 3°C/min to separate methyl esters ranging from 12 to 22 carbon fatty acids. Retention time and peak areas were determined electronically using a Hewlett-Packard Recording Integrator. Identification of methyl esters was by comparison with known methyl ester standards. All results were tested for statistical significance by Student's *t*-test compared with the respective controls.

RESULTS

Incorporation and conversion of [2-^{14}C]eicosa-8,11,14-trienoic acid into arachidonic acid in Sertoli and Leydig cells. Figure 1 shows how increasing concentrations of the substrate modified the steady-state level of incorporation of labeled eicosatrienoic acid and its subsequent transformation into arachidonic acid by Sertoli (panel A) and Leydig (panel B) cells. Precursor acid was taken up by both types of cells. The amount of label incorporated after 3 h incubation followed a hyperbolic curve that became saturated at approximately 15 μM. The uptake of 20:3n-6 by the Leydig cells increased progressively up to 7.5 μM substrate. A similar pattern was obtained for Sertoli cells when the substrate concentration reached 5 μM. After this point the rate of incorporation increased more slowly, and at the highest concentrations of substrate studied (40 μM), the amount of fatty acid incorporated was similar in both cell types.

Arachidonic acid was not further elongated and/or desaturated as indicated by the absence of significant amounts of label in total cellular higher PUFA methyl esters. In all experiments, the only labeled acid found in the incubation medium was 20:3n-6, indicating that the arachidonic acid formed in the cells was fully integrated into complex membrane lipids. Approximately 15% of the eicosatrienoic acid incorporated was transformed into arachidonic acid. The amount of 20:4n-6 formed by Leydig cells was nearly twice that synthesized by Sertoli cells.

The time curves of incorporation and desaturation of eicosatrienoic acid by Sertoli and Leydig cells incubated with 15 μM substrate are shown in Figure 1 (panels C and D). In both types of cells, 20:3n-6 was rapidly incorporated

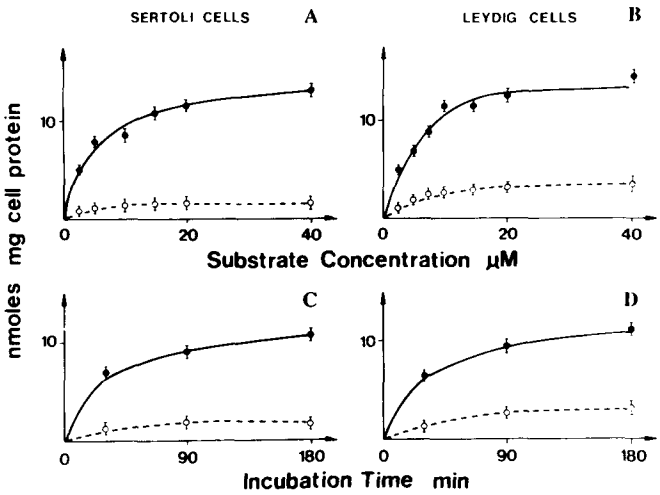

FIG. 1. Incorporation of [2-^{14}C]eicosa-8,11,14-trienoic acid (closed circles) and desaturation to arachidonic acid (open circles), as a function of substrate concentration (upper panel) and incubation time (lower panel) in Sertoli (left) and Leydig (right) cells isolated from control rats. Values are the mean ± SEM of four incubations. The incubation time used in the upper panel (A and B) was 180 min, while the concentration of the substrate added in the experiments showed in the lower panel (C and D) was 15 μM. Other incubation conditions are described in the Materials and Methods section.

during the initial 30 min of incubation. The incorporation reached a plateau soon thereafter. In Sertoli and Leydig cells, eicosatrienoic acid was partially converted into arachidonic acid, about 15% and 25% respectively; the values remained constant after 30 min of incubation.

Effect of in vivo *administration of ACTH on the incorporation and desaturation of [2-^{14}C]eicosa-8,11,14-trienoic acid into arachidonic acid in isolated Sertoli and Leydig cells.* Incorporation of labeled 20:3n-6 into the Sertoli or Leydig cells isolated from ACTH-treated rats was similar to that seen in the same cell types isolated from non-hormonally-treated animals (Fig. 2). By contrast, the transformation of 20:3n-6 into 20:4n-6 was decreased by 48% and 40%, respectively, in Sertoli and Leydig cells after prior treatment of the animals with ACTH.

Effect of addition of ACTH and diBucAMP to the incubation medium of isolated Sertoli and Leydig cells obtained from untreated rats. To test whether the inhibitory effect of ACTH on the biosynthesis of arachidonic acid also was produced in cells isolated from untreated rats, the hormone was added to the incubation medium. Figure 3 shows the incorporation and desaturation of [2-^{14}C]-eicosa-8,11,14-trienoic acid in the isolated cells 210 min after the addition of ACTH to the medium. We also tested the effect of addition of diBucAMP to the incubation medium under the same conditions. Neither the hormone nor the cyclic nucleotide modified the incorporation of eicosatrienoic acid into either cell type. However, both agents caused a significant decrease in arachidonic acid biosynthesis in both Sertoli and Leydig cells as compared with replicate cells incubated in the absence of ACTH and diBucAMP (Fig. 3).

Effect of addition of corticosterone to the incubation medium of Sertoli and Leydig cells isolated from untreated rats. Figure 4 shows the effect of the hormone or its vehicle on the incorporation and transformation of

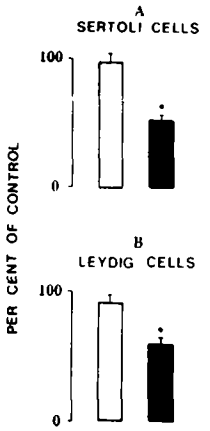

FIG. 2. Effect of ACTH administered to intact rats on the incorporation (open columns) and desaturation (filled columns) of [2-^{14}C]eicosa-8,11,14-trienoic acid in Sertoli (panel A) and Leydig (panel B) cells after 3 h of incubation. One hundred percent corresponds to control values: 10.02 ± 0.015 nmol/mg protein incorporated and 2.006 ± 0.102 nmol/mg protein desaturated by Sertoli cells (A); and 15.22 ± 0.63 nmol/mg protein incorporated and 2.292 ± 0.150 nmol/mg protein desaturated by Leydig cells (B). The incubation conditions were as described in the Materials and Methods section. Values are the mean of four incubations ± SEM. Asterisk represents significant difference relative to the respective control $P < 0.001$.

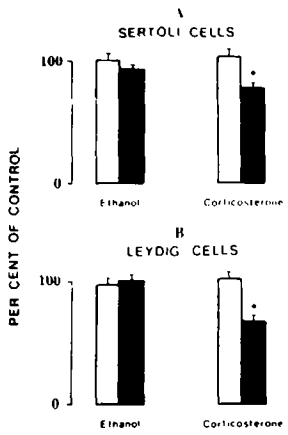

FIG. 4. Effect of the addition of corticosterone or its vehicle to the incubation medium on the incorporation (open columns) and desaturation (filled columns) of [2-^{14}C]eicosa-8,11,14-trienoic acid in isolated Sertoli (A) and Leydig (B) cells obtained from untreated rats. One hundred percent corresponds to 10.94 ± 0.30 nmol/mg protein incorporated and 2.189 ± 0.053 nmol/mg protein desaturated by Sertoli (A); and 10.39 ± 0.16 nmol/mg protein incorporated and 2.145 ± 0.195 nmol/mg protein desaturated by Leydig (B) cells. The incubation conditions were as described in the Materials and Methods section. Values are the mean of four incubations ± SEM. Asterisk indicates a significant difference from the respective controls $P < 0.01$.

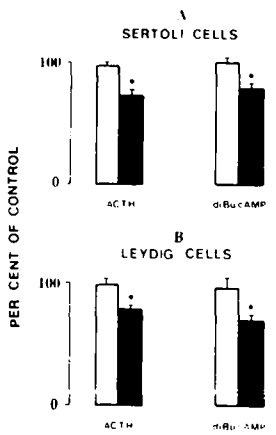

FIG. 3. Effect of addition of ACTH (250 μ IU/per flask) and diBucAMP (0.5 mM) to the incubation medium on the incorporation (open columns) and desaturation (filled columns) of [2-^{14}C]eicosa-8,11,14-trienoic acid in Sertoli (panel A) and Leydig (panel B) cells obtained from untreated rats. One hundred percent corresponds to 10.48 ± 0.020 nmol/mg protein incorporated and 2.094 ± 0.076 nmol/mg protein desaturated by Sertoli cells (A); and 13.08 ± 0.27 nmol/mg protein incorporated and 2.207 ± 0.172 nmol/mg protein desaturated by Leydig cells (B). The incubation conditions were described in the Materials and Methods section. Values are the mean of four incubations ± SEM. Asterisk indicates a significant difference from the respective controls $P < 0.01$.

[2-^{14}C]eicosa-8,11,14-trienoic acid in Sertoli (Fig. 4A) and Leydig (Fig. 4B) cells after 210 min of incubation. Corticosterone inhibited the production of arachidonic acid by both types of cells in the absence of any change in substrate incorporation. Ethanol modified neither the incorporation nor the transformation of eicosatrienoic acid (Fig. 4).

Effect of ACTH on the fatty acid composition of total lipids in Sertoli and Leydig cells. The relative percentages of total fatty acids of Sertoli cells obtained from untreated rats either not incubated or incubated for 3 h in the presence of 20:3n-6 are shown in Table 1. In the untreated cells, the major components in the non-essential fatty acid series were 16:0 and 18:1 (approximately 20%), while in the essential fatty acid series 22:5n-6 and 20:4n-6 were most abundant. After 3 h of incubation in the presence of the labeled precursors the only significant difference observed was a small increase in 20:3n-6.

ACTH treatment of the rats produced significant changes in the fatty acid composition of the Sertoli cells when compared to cells obtained from untreated animals. The relative abundance of 16:1 and 18:2n-6 increased, while the proportions of 20:4n-6, 22:5n-6, 22:5n-3 and 22:6n-3 decreased markedly. In contrast, addition of ACTH, corticosterone or diBucAMP *in vitro* produced no significant differences in the total fatty acid composition when compared to cells incubated in the absence of either the hormones or the nucleotide. No significant changes in the total amount of fatty acids were observed among the different groups. The values for the control rats were 23.1 ± 0.8 μg/10^6 cells.

The total fatty acid profile of the non-incubated Leydig cells indicated that 16:0, 18:0, 18:1 and 20:4n-6 acids were the major components, while the percentage of 22:5n-6 was approximately 2-3%. The results agree with those published (6) and, thus, are not shown here. Although 3 h of incubation produced a small, though significant, increase in 20:3n-6, the other fatty acids remained unchanged.

The administration of ACTH to the rats and the addition of ACTH or diBucAMP to the incubation medium did not produce significant changes in the amount or composition of total fatty acids of Leydig cells. The total

TABLE 1

in vivo and *in vitro* Effect of ACTH on the Total Fatty Acid Composition of Sertoli Cells[a]

Fatty acid	Non-incubated control (%)	Incubated control (%)	*in vitro* ACTH (%)	*in vitro* diBucAMP (%)	*in vitro* corticosterone (%)	*in vivo* ACTH (%)
16:0	23.7 ± 0.6	22.5 ± 0.6	23.5 ± 0.1	22.8 ± 0.4	22.7 ± 0.4	25.2 ± 1.1
16:1	1.7 ± 0.6	1.4 ± 0.0	2.0 ± 0.2	2.2 ± 0.1	2.1 ± 0.2	3.0 ± 1.1[b]
18:0	10.5 ± 0.2	10.9 ± 1.1	9.9 ± 0.3	10.0 ± 0.4	10.3 ± 0.5	13.2 ± 1.1
18:1	19.7 ± 0.6	19.7 ± 0.6	19.3 ± 1.1	20.1 ± 0.3	19.7 ± 0.5	22.4 ± 0.9
18:2n-6	4.3 ± 0.3	3.4 ± 0.6	3.9 ± 0.1	4.0 ± 0.3	3.7 ± 0.3	6.8 ± 0.6[b]
20:3n-6	1.4 ± 0.2	3.1 ± 0.1[c]	2.9 ± 0.1	3.0 ± 0.0	3.0 ± 0.0	2.6 ± 0.0[b]
20:4n-6	14.4 ± 1.4	12.8 ± 0.1	12.2 ± 0.0	12.2 ± 0.0	12.3 ± 0.1	10.1 ± 0.5[b]
22:4n-6	2.6 ± 0.1	2.9 ± 0.1	2.3 ± 0.0	2.6 ± 0.2	2.7 ± 0.1	1.9 ± 0.0[b]
22:5n-6	15.4 ± 1.7	16.1 ± 0.4	17.1 ± 0.3	16.5 ± 0.3	16.3 ± 0.3	12.5 ± 0.5[b]
22:5n-3	3.2 ± 0.4	4.1 ± 0.1	4.1 ± 0.5	3.8 ± 0.8	4.0 ± 0.5	1.3 ± 0.3[b]
22:6n-3	3.1 ± 0.4	3.1 ± 0.1	2.8 ± 0.1	2.8 ± 0.2	3.0 ± 0.2	1.0 ± 0.2[b]
20:4n-6/18:2n-6	3.4	3.8	3.1	3.1	3.3	1.5

[a]Each value represents the mean of four incubations ± 1 SEM.

[b]$P < 0.01$ *vs.* incubated control.

[c]$P < 0.001$ *vs.* non-incubated control.

amount of fatty acids in the control group was 23.9 ± 1.1 $\mu g/10^6$ cells. For this reason, the results are not reported here.

DISCUSSION

The testis is a steroidogenic tissue which consists of a heterogeneous population of cell types. Most previous studies on the biosynthesis of polyunsaturated fatty acids in testis were done without accounting for the metabolic peculiarities of the individual cell types. In the present studies we have used isolated cells, because they are valuable models in which environmental conditions can be controlled more rigidly and complex interactions between the cells can be minimized. The synthesis of arachidonate from linoleate has previously been documented in primary cultures of Sertoli cells from young rats in which both Δ6 and Δ5 desaturating activities work in concert (10). Our experiments have further characterized the desaturase activity of these cells and of Leydig cells by demonstrating the conversion of eicosa-8,11,14-trienoic acid to arachidonate. Hence, an active complex of Δ5 desaturase appears to be operative in both cell types. The Δ5 desaturase activity in the testicular cells differs from that in liver and in other steroidogenic tissues, such as the adrenal gland (35). The amount of 20:3n-6 transformed into 20:4n-6 is higher in adrenocortical cells (35) than in Sertoli or Leydig cells (Fig. 1). Adrenocortical cells transformed 50% of the fatty acid that was incorporated into arachidonate, while in testicular cells the percentage was less than 20%. The amount of 20:3n-6 incorporated in both types of testicular cells was reflected in a significant increase in the relative percentage of 20:3n-6, as is apparent in the fatty acid profile (Table 1). Considering that these cell types have high levels of arachidonic acid and that the amount of 20:3n-6 that was further desaturated was about 20% of that incorporated, an increase in the relative percentage of 20:4n-6 was not expected after only 3 h of incubation. Such observations are consistent with a relatively sluggish Δ5 desaturase activity in the two cell types. Under similar experimental conditions, either hepatocytes or adrenocortical cells showed both a lesser increase in eicosatrienoic acid incorporation and a more pronounced enhancement in arachidonic acid synthesis when compared to the isolated testicular cells (see Fig. 1, this paper; also Fig. 1, ref. 35, and Figs. 1 and 3, ref. 36). In all these separate studies with cell cultures from the three tissues, the radioactive precursor was the same fatty acid (*i.e.*, 20:3n-6). Therefore, differences in arachidonic acid formation appear to be due to a more pronounced Δ5 desaturase activity in the adrenal and liver than in the testicular cells.

PUFA biosynthesis is regulated by the hypophysis through the production of ACTH. This was previously demonstrated in various tissues, including the liver and the adrenal gland (21). The results obtained in this work showed that ACTH also diminishes the biosynthesis of arachidonic acid in Sertoli and Leydig cells (Figs. 2 and 3). This effect was apparent when rats were treated with ACTH, or when the hormone was added to the incubation medium of the isolated testicular cells. The inhibition produced by the hormone *in vivo*, however, was greater than that evoked in isolated cells. The *in vivo* effect was reflected accordingly in the fatty acid composition of Sertoli cells. In order to more accurately assess the activity of the arachidonate-synthetic pathway, we analyzed the 20:4/18:2 ratio in these cells and found a 50% decrease in the cells isolated from the hormone-treated rats. In hepatocytes and adrenal cells, ACTH produced an inhibition in both Δ6 and Δ5 desaturating activities (21). The inhibition of the former also can be inferred from the fatty acid composition; we found an accumulation of 18:2n-6 and a diminution in the amount of 20:3n-6 (Table 1) after hormone treatment. Control Sertoli cells before incubation contained 1.4% 20:3n-6. After 3 h of incubation, the relative percentage of 20:3n-6 increased to 3.1%, but was decreased by 16% when Sertoli cells were isolated from ACTH-treated rats. Since this decrease occurred even when the precursor 18:2n-6 accumulated after hormonal intervention, the depression observed in the level of 20:3n-6 *in vivo* must depend on a lesser conversion of 18:2n-6 to 18:3n-6 in addition to diminution in 20:3n-6 desaturation that we measured directly. Moreover, the low

percentages observed in 22:5n-3 and 22:6n-3 also are an indirect indication of a depression in Δ4 desaturase activity. Similar results were obtained when the effect of ACTH on the production of higher homologs formed from α-linolenic acid was studied in adrenocortical cells (37).

In contrast to Sertoli cells, the total fatty acid composition of Leydig cells showed no significant changes after either *in vivo* or *in vitro* exposure to the hormone in spite of the significant depression observed in Δ5 desaturation activity (Figs. 2 and 3).

The most common signal-transducing system mediating ACTH stimulation involves cAMP. In the present work, we have shown that the addition of diBucAMP to the incubation medium of Sertoli and Leydig cells mimicked the inhibition produced by ACTH on the formation of arachidonic acid (Fig. 3). This may indicate that cAMP could well be a second messenger for the action of ACTH on polyunsaturated fatty acid biosynthesis in testicular cells. The inhibition produced by either ACTH or diBucAMP *in vitro* was smaller than that evoked when the hormone was injected directly into the rats. This quantitative discrepancy may imply that at least one other effector participates in the depression of Δ5 desaturation.

Glucocorticoids directly inhibit the biosynthesis of arachidonic acid in several tissues (21,38,39). On the other hand, it is well-known that ACTH increases the plasma levels of corticosterone several-fold. This hormone, in turn, could potentiate the *in vivo* effect of ACTH on Δ5 desaturating activity. We found that the addition of corticosterone to the incubation medium blocked the biosynthesis of arachidonic acid in both Sertoli and Leydig cells (Fig. 4). We thus conclude that the higher inhibition of polyunsaturated fatty acid biosynthesis observed after *in vivo* treatment with ACTH may arise, at least in part, from a concerted effect of both hormones.

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Glucocorticoid and Mineralocorticoid Hormones Depress Liver Δ5 Desaturase Activity Through Different Mechanisms

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The effects of 11-deoxycorticosterone and aldosterone on liver Δ5 desaturase activity were examined. Both steroid hormones significantly depressed the conversion of [1-^{14}C] eicosatrienoic acid to arachidonic acid. However, the mechanism of action of each of these hormones was different. The effect of 11-deoxycorticosterone was mediated by a soluble protein present in the liver cytosolic fraction. The biological activity of this protein, having a molecular weight lower than 25 kDa, was impaired by trypsin digestion. To determine whether the inhibitory protein was induced through glucocorticoid or mineralocorticoid receptor occupancy, cultured Morris minimal deviation hepatoma cells were pre-treated with the antiglucocorticoid cortexolone or the mineralocorticoid receptor antagonist spironolactone. The results obtained demonstrated that only glucocorticoid receptor structures were involved in the induction of this regulatory protein. The inhibitory response evoked by aldosterone was mediated by a different mechanism. In the case of aldosterone, the inhibitory action affected the microsomal membranes and was not mediated by a soluble protein messenger.

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Polyunsaturated fatty acid biosynthesis in liver is regulated by glucocorticoids. The administration of these hormones to rats has been shown to increase Δ9 desaturase activity (1) and to decrease Δ6 and Δ5 desaturase activities (2,3). In addition, the incubation of Morris minimal deviation hepatoma cells (HTC), or of hepatocytes from normal rats with dexamethasone, provoked a strong depression in the conversion of eicosatrienoic acid to arachidonic acid (4,5). However, the regulatory mechanisms of fatty acid biosynthesis are still poorly understood.

We have demonstrated that the regulatory action of glucocorticoid hormones on the fatty acid desaturase activities depends on a protein factor whose biosynthesis is brought about by hormonal induction (1,3). Although 11-deoxycorticosterone (DOC) does not have an 11-β-hydroxy group, which is thought to be essential for glucocorticoid activity (6), we have previously demonstrated that in HTC cells this hormone can induce a soluble regulatory factor that stimulates Δ9 desaturase activity (7). Two species of adrenocortical hormone receptors have been defined: i) Those receptors associated with mineralocorticoid function which have a high affinity for aldosterone (ALDO) and a lower affinity for corticosterone (8); and ii) those receptors associated with glucocorticoid function which have a high affinity for corticosterone and a lower affinity for aldosterone (9,10).

The aim of the present study was to assess the effectiveness of DOC or aldosterone in depressing liver Δ5 desaturase activity. In addition, we set out to determine whether induced inhibition of Δ5 desaturase was specific for glucocorticoid receptor occupancy. Taking into account that DOC can exert glucocorticoid hormone-type effects (11), we also compared the ability of DOC and aldosterone to induce the above-mentioned inhibitory protein factor in the presence or absence of cortexolone or spironolactone. The compounds were chosen because they are antagonists for gluco- and mineralocorticoid receptors, respectively (12,13).

MATERIALS AND METHODS

Chemicals. Radioactive [1-^{14}C]eicosa-8,11,14-trienoic acid (59.0 mCi/mmol, 98% radiochemically pure) was purchased from New England Nuclear (Boston, MA). Unlabeled eicosatrienoic acid was purchased from NuChek Prep (Elysian, MN). NADH, ATP, coenzyme A (CoASH) and other co-factors, HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), bovine serum albumin, trypsin type III, trypsin soybean inhibitor type I, delipidated bovine serum albumin, 11-deoxycorticosterone (hemisuccinate) (DOC), ALDO, spironolactone and cortexolone (Reichstein substance S 21-acetate) were from Sigma Chemical Co. (St. Louis, MO). Dexamethasone (sodium phosphate salt) was from Merck, Sharp and Dhome (Buenos Aires, Argentina).

Animals and their treatment. Adult female Wistar rats weighing 160–180 g were housed two per cage in a thermostatically controlled room at 22 ± 2°C. Food (Cargill type "C", Rosario, Argentina) and water were available *ad libitum*. Cargill standard pelleted diet, type "C", consisted of (in calories): 56.7% carbohydrates, 10.4% lipids and 32.9% protein, vitamins and minerals. The fatty acid relative percentages of this diet were 21.4% palmitic,2.1% palmitoleic, 8.2% stearic, 24.9% oleic, 37.7% linoleic and 0.2% arachidonic. The rats were divided in groups of four animals each. One group was injected intraperitoneally with a single dose of DOC (1.0 μmol/kg body weight). Another group of rats was injected with ALDO (1.0 μmol/kg body weight), and the last group (control rats) was injected only with the vehicle in which the hormone was dissolved (ethanol, 20 mM). All rats were killed by decapitation without anesthesia 24 h after the injection. In order to avoid individual differences among the nutritional status of the animals, the rats were fasted for 24 h, fed for 2 h, and then killed 12 h after the end of the feeding period.

Isolation of crude microsomes. Livers (processed individually) were rapidly excised and immediately placed

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Abbreviations: ALDO, aldosterone; CoASH, coenzyme A (sodium salt); DOC, 11-deoxycorticosterone (hemisuccinate); Dx, dexamethasone (sodium phosphate salt); GLC, gas-liquid chromatography; H, Sp fraction containing proteins with MW higher than 25 kDa; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; HTC, Morris minimal deviation hepatoma cells number 7288 c; IMEM-Zo, improved minimal essential medium-zinc option; L, Sp fraction containing proteins with MW lower than 25 kDa; M, crude liver microsomes; Me, extracted liver microsomes; Sp, soluble proteins obtained by washing crude microsomes; Spa, specific activity; T-Sp, Sp fraction proteolized by incubation with trypsin.

in ice-cold homogenizing solution (14). The excised livers were homogenized in 3 mL of homogenizing medium for each gram of liver and centrifuged at 10,000 × *g* for 20 min at 2–4°C. The pellet was discarded and the supernatant was centrifuged again (110,000 × *g* for 60 min) at 1–2°C. The supernatant from the second centrifugation was used as cytosol fraction. The pellets were resuspended in cold homogenizing solution up to a final protein concentration of 50 mg/mL, and were used as crude microsomes from DOC-treated (M_{DOC}), ALDO-treated (M_{ALDO}) or control (M_C) rats.

Microsome extraction. In some experiments, extracted microsomes from rat liver (Me) were obtained following the procedure described previously (15). Briefly, crude microsomes from control or hormonally-treated rats in the proportion of 5 mg protein per 3 mL of standard extraction solution (15) were shaken at 0–2°C under air for 15 min. The standard extraction solution contained 0.25 M sucrose, 0.04 M phosphate buffer (pH 7.40), 0.04 M NaCl, 0.15 M KCl, 0.004 M *N*-acetylcysteine, 0.04 M NaF, 0.3 mM nicotinamide and 15 mM $MgCl_2$. The suspension was then centrifuged at 110,000 × *g* (1–2°C) for 60 min in a L-2-65 ultracentrifuge (Beckman Instruments, Palo Alto, CA). Two fractions, one corresponding to the extracted microsomes (Me) and the other to the supernatant soluble fractions (Sp), were obtained. Sp fractions obtained by washing microsomal suspensions from control or hormonally-treated rats routinely contained 40–45% of proteins associated with crude microsomal fractions.

Hepatoma cultured cells. Culture cells used in these experiments came from Morris minimal deviation hepatoma number 7288 c, a solid hepatoma from which the ascites tumor line (HTC cells) had been derived (16). HTC cells were maintained and grown *in vitro* at 37°C in confluent layer attached to glass on Swim's 77 (S-77) medium supplemented with 10% (vol/vol) calf serum, using conventional sterile conditions, as previously described (17).

Cytosol fractions from control or hormone-treated HTC cells. When HTC cells were in logarithmic phase (approximately 72 h), Swim's 77 was replaced by Richter's improved minimal essential medium-zinc option (IMEM-Zo) (18). In the first series of experiments, several culture flasks were supplemented with DOC, dexamethasone (Dx) or ALDO at a final concentration of 10^{-6}M. In some experiments the cells were maintained under these conditions for 12 h, harvested as indicated above, processed separately (control, DOC, Dx or ALDO-treated cells) and pelleted by centrifugation at 4°C (1,000 × *g* for 10 min). Each pellet was washed three times with 10 mL of cold, steroid-free IMEM-Zo medium and resuspended in 5 mL of the same ice-cold homogenizing solution use for the isolation of liver microsomes (14). The cells were homogenized immediately in a small, stainless-steel hand homogenizer and centrifuged at 110,000 × *g* for 60 min. The supernatants obtained were used as cellular (control or hormone-treated) cytosol fractions. In a second series of experiments, cytosol fractions of steroid-treated HTC cultures were prepared following the procedures described above, except that the cells were incubated with DOC (10^{-6}M) for 12 h, after pre-incubation or not with either cortexolone (10^{-4}M) or spironolactone (10^{-4}) for 2 h.

Isolation of light (L) and heavy (H) fractions from microsomal soluble proteins (Sp). Aliquots (4 mL) of soluble proteins (Sp) extracted from microsomes of control or DOC-treated rats were applied, without further manipulation, to Centriflo CF-254 filter cones (Amicon Co., Lexington, MA) and centrifuged in the cold at 1,600 × *g* for 55 min. The ultrafiltrates of Sp_C or Sp_{DOC} were collected separately and used as L-control or L-DOC Sp fractions. These fractions contained 95% of the soluble proteins with molecular weights of 25 kDa or less. The ultrafilter cones were rinsed three times (2 mL each time) with cold homogenizing solution, and the filtrates obtained after centrifugation were discarded. The remaining protein fractions in the cones were resuspended in homogenizing medium (3 mL) and used as H-control or H-DOC Sp fractions. These fractions contained proteins with molecular weights higher than 25 kDa.

Fatty acid desaturase activity assay. Δ5 Desaturase activity was measured by estimation of the percentage conversion of [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. Liver microsomal protein (5 mg) was incubated with 100 nmol of a mixture of labeled and unlabeled eicosatrienoic acid (0.25 μCi/tube) at 37°C for 10 min in a shaking water bath. The incubation medium (1.5 mL) contained the same solution used for the extraction of microsomes except that in the desaturase activity assay 3.5 mM ATP, 0.2 mM CoASH and 1.5 mM NADH were added.

Under these experimental conditions the desaturase enzyme was completely saturated with the substrate (19). In some experiments, cytosol fractions from steroid-treated HTC cells or Sp fractions from microsomes of hormone-injected rats were added to the incubation medium (0.60 mg protein per tube). In all cases the final volume of incubation was 1.5 mL. The reaction was started by addition of an aliquot of crude of extracted liver microsomal suspension. It was stopped by addition of 2 mL of 10% (w/vol) KOH in ethanol plus 500 μL/tube methanol to facilitate the subsequent extraction step. The fatty acids were recovered after saponification of the incubation mixture, and were methylated as described elsewhere (1).

Radioactivity of the recovered methyl esters (> 98%) was determined in a Beckman liquid scintillation counter (model LS-3133, Beckman Instruments, Palo Alto, CA) with 96% efficiency for ^{14}C. Distribution of radioactivity among the fatty acid methyl esters was measured by gas-liquid chromatography (GLC) as detailed in a previous study (5). The chromatographic peaks were identified by comparison of the relative retention times with those of standards chromatographed under the same conditions. Percentage conversion was calculated from the radioactivity distribution between substrate and product, and was measured directly on the radiochromatogram (20). The column was packed with 10% SP-2330 on Chromosorb WAW-DMCS (100–200 mesh) (Supelco Inc., Bellefonte, PA).

Trypsinization of soluble fractions. A solution of 6 mg trypsin (type III) in 0.25 mL phosphate buffer (pH 7.4) was added to each 6 mL Sp corresponding to 0.6 mg protein per mL, and the mixture was incubated for 60 min at 37°C. Proteolysis was stopped by the addition of 12 mg trypsin soybean inhibitor (type I).

RESULTS

Table 1 shows the changes that occurred in liver Δ5 desaturase activity 24 h after the injection of 1 μmol/kg

TABLE 1

Effect of Steroid Treatment on Microsomal Rat Liver Δ5 Desaturase Activity

Microsomes[a]	Δ5 Desaturase activity: Spa = nmol desat. / min•mg/prot.	% Change compared to control
M_C	0.58 ± 0.02[b]	
M_{DOC}	0.22 ± 0.02[c]	62 ± 3
M_{ALDO}	0.19 ± 0.03[c]	69 ± 5

[a]Five mg of microsomal protein from control (M_C) and deoxycorticosterone (M_{DOC})- or aldosterone (M_{ALDO})-treated rats were incubated at 37°C for 10 min in a shaking water bath with 100 nmol of a mixture of labeled and unlabeled eicosatrienoic acid (0.25 μCi/tube).

[b]The results are given as the means of four animals per group ± SEM.

[c]Determined significantly different from M_C using Student's *t*-test ($P < 0.001$).

TABLE 2

Effect of the Addition of Soluble Proteins (Sp) or Cytosol to Rat Liver Microsomes on Δ5 Desaturase Activity[a]

	Crude microsomes (M)		
	M_C	M_{DOC}	M_{ALDO}
None	29.6 ± 1.0[b]	14.9 ± 0.8	18.7 ± 1.1
	Extracted microsomes (Me)		
	Me_C	Me_{DOC}	Me_{ALDO}
None	20.7 ± 0.8	10.3 ± 0.6	8.1 ± 0.3
Sp_C	28.6 ± 0.9	15.4 ± 0.7	20.4 ± 0.2
Sp_{DOC}	13.7 ± 0.9	4.8 ± 0.3	—
Sp_{ALDO}	28.6 ± 0.7	—	21.2 ± 0.8
$Cytosol_C$	29.0 ± 0.7	16.1 ± 0.8	21.8 ± 0.5
$Cytosol_{DOC}$	12.0 ± 0.5	3.3 ± 0.5	—
$Cytosol_{ALDO}$	26.9 ± 0.5	—	18.9 ± 1.0

[a]Soluble proteins (0.6 mg protein/tube) or cytosol (0.6 mg protein/tube) from control (C), DOC-treated (DOC) or ALDO-treated (ALDO) rats were added to crude (M) or extracted (Me) microsomal (5 mg of microsomal protein) in 1.5 mL of incubation medium. For details see Materials and Methods.

[b]The results are the mean of three determinations expressed as percent conversion of eicosatrienoic acid to arachidonic acid ± SEM. Incubation conditions were as described in Table 1.

body weight of DOC or ALDO into rats. Both hormones significantly decreased the conversion of eicosatrienoic acid to arachidonic acid.

A number of experiments were designed in order to establish whether the mechanism of DOC or ALDO action is mediated by the induction of a regulatory protein present in the cytosolic fraction of the cells. In the first one, the effect on Δ5 desaturase activity caused by the addition of liver microsome soluble fractions from control or DOC-injected rats to crude microsomes was determined. As shown in Table 2, liver Δ5 desaturase activity was strongly depressed by extraction of microsomes (from control or DOC-treated rats) with a low ionic strength solution, but in control microsomes the depression observed was considerably higher.

The addition of 0.6 mg of soluble fraction obtained from control livers (Sp_C) to extracted microsomes of untreated animals (Me_C) restored almost 100% of Δ5 desaturase activity. A similar effect was observed with the addition of liver cytosol from control rats. However, soluble protein and cytosol obtained from DOC-treated animals were unable to restore the desaturation capacity of washed microsomes (Me_C or Me_{DOC}), instead they significantly decreased enzyme activity.

When extracted microsomes from hormonally-treated rats (Me_{DOC}) were incubated with soluble fractions or cytosol obtained from control rats, Δ5 desaturase activity increased to values observed with whole microsomes from DOC-injected animals. Conversely, when extracted soluble protein or liver cytosol from hormonal treated animals was added to Me_{DOC}, the conversion of eicosatrienoic acid to arachidonic acid was significantly depressed and Δ5 desaturase activity was the lowest measured. Soluble proteins or liver cytosol fractions were added to complete or extracted microsomes from control or ALDO-treated animals in similar experiments (Table 2). DOC strongly depressed the conversion of eicosatrienoic acid to arachidonic acid both in complete or in extracted-liver microsomes.

The addition of 0.6 mg protein of soluble fraction from control liver (Sp_C) to extracted microsomes of untreated animals (Me_C) restored the Δ5 desaturase activity to values obtained with crude microsomes from control rats. A similar effect was observed with added soluble protein fractions from aldosterone-treated rats (Sp_{ALDO}) or cytosolic fractions from control or aldosterone-injected animals.

When extracted microsomes from hormonally-treated rats (Me_{ALDO}) were incubated with soluble fractions from control or aldosterone-injected animals (Sp or cytosol), Δ5 desaturase activity only increased to values observed with crude microsomes from treated animals (M_{ALDO}). Therefore, the conversion of eicosatrienoic acid to arachidonic acid in liver microsomes from aldosterone-injected rats was significantly depressed (as compared to control animals), even after the addition of the different soluble fractions tested.

Results from the incubation of [1-^{14}C]eicosatrienoic acid with control crude microsomes or control-extracted microsomes plus cellular cytosolic fractions of control or hormonally-treated isolated cells are shown in Table 3. When the 110,000 × *g* supernatant from homogenates of control or ALDO-treated HTC cells was added to extracted liver microsomes (Me_C), Δ5 desaturase activity that was previously reduced by washing microsomes with a low ionic strength solution, increased. Conversely, the addition of cytosolic fractions from Dx- or DOC-treated HTC cells to extracted microsomes from control rats, inhibited rather than restored Δ5 desaturating capacity.

The effect of trypsin digestion of soluble fractions from control and DOC-treated rats on Δ5 desaturase activity is shown in Table 4. Proteolysis inhibited the capacity of the control soluble fraction to reactivate the fatty acid desaturation of extracted microsomes from control animals. The ability to reactivate the Δ5 desaturase reaction was not impaired when the control soluble fraction was treated with trypsin that had previously been inactivated by trypsin inhibitor. As shown in Table 3, the soluble fraction from DOC-treated animals significantly reduced the Δ5 desaturase capacity of Me_C. This inhibition was stronger than that produced by washing crude micro-

TABLE 3

Effect of the Addition of Cytosol from Hormonally-Treated HTC Cells to Rat Liver Microsomes on Δ5 Desaturase Activity[a]

	% Conversion 20:3n-6 to 20:4n-6
Crude microsomes (M_C	31.5 ± 0.9[b]
Extracted microsomes (Me_C	20.8 ± 0.5
Me_C + cytosol from control HTC cells	28.7 ± 0.8
Me_C + cytosol from DOC-treated HTC cells	15.6 ± 0.4
Me_C + cytosol from ALDO-treated HTC cells	29.1 ± 0.6
Me_C + cytosol from Dx-treated HTC cells	13.0 ± 0.3

[a]Cytosol fraction from control (C), deoxycorticosterone (DOC) or aldosterone (ALDO)-treated HTC cells, were added (0.6 mg protein/tube) to 5 mg of extracted liver microsomal protein (Me) from control rats. Final incubation medium was 1.5 mL. See Materials and Methods for experimental details.

[b]Data are expressed as percent conversion of [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid and are given as mean of three individual determinations ± SEM. Analyses of the samples were done as described in Materials and Methods.

TABLE 4

Effect of Trypsin-Treated Soluble Fractions on Δ5 Desaturase Activity of Extracted Microsomes from Control (C) Rats[a]

	% Conversion 20:3n-6 to 20:4n-6
Crude microsomes from control rats (M_C)	35.8 ± 1.7
Extracted microsomes from control rats (Me_C)	15.1 ± 1.3
Me_C + Sp_C	31.2 ± 1.8
Me_C + T-Sp_C	16.2 ± 1.5
Me_C + Sp_C + (trypsin + trypsin inhibitor)[b]	30.9 ± 2.2
Me_C + Sp_{DOC}	9.7 ± 0.7
Me_C + T-Sp_{DOC}	15.6 ± 1.4
Me_C + Sp_{DOC} + (trypsin + trypsin inhibitor)[b]	9.8 ± 1.2

[a]Desaturation activity was measured by incubation of 5 mg of liver extracted microsomal protein (Me_C) plus 0.6 mg per tube of soluble proteins from control (Sp_C) or 11-deoxycorticosterone-injected (Sp_{DOC}) rats. In some tubes trypsin-treated Sp fractions (T-Sp) from control or DOC-injected rats were added (0.6 mg/tube).

[b]Sp treated with trypsin previously inhibited by trypsin inhbitor. In all cases, the final volume of incubation was 1.5 mL. The results are expressed as percent conversion of eicosatrienoic acid to arachidonic acid and are the mean of three individiual determinations ± 1 SEM.

TABLE 5

Effect of Addition of the H-Cytosolic or L-Cytosolic Fractions from Control (C), Deoxycorticosterone (DOC)-Treated Rats to Extracted Liver Microsomes (Me) from Control Rats, on Δ5 Desaturase Activity[a]

	% Conversion 20:3n-6 to 20:4n-6
Crude microsomes (M_C)	32.3 ± 1.1
Extracted microsomes (Me_C)	20.1 ± 0.8
Me_C + H-Sp fraction from control rats	33.3 ± 0.6
Me_C + H-Sp fraction from DOC-treated rats	30.9 ± 1.3
Me_C + L-Sp fraction from control rats	29.8 ± 0.5
Me_C + L-Sp fraction from DOC-trated rats	14.0 ± 0.3

[a]H- and L-fractions were prepared by ultracentrifugation. For details see Materials and Methods. Data are expressed as percent conversion of [1-^{14}C]eicosatrienoic acid to arachidonic acid and are the mean of three determinations ± SEM.

TABLE 6

Effect of Pre-Treatment of HTC Cells with the Antiglucocorticoid Cortexolone or the Antimineralocorticoid Spironolactone on the Action of Deoxycorticosterone (DOC) on Eicosatrienoic Acid Desaturation[a]

	% Conversion 20:3n-6 to 20:4n-6
Crude microsomes from control rats (M_C)	29.8 ± 1.3
Extracted microsomes from control rats (Me_C)	18.5 ± 0.9
Me_C + cytosol from control HTC cells	27.6 ± 0.8
Me_C + cytosol from cortexolone-treated HTC cells	25.8 ± 1.1
Me_C + cytosol from spironolactone-treated HTC cells	25.4 ± 1.4
Me_C + cytosol from DOC-treated HTC cells	11.0 ± 1.2
Me_C + (cortexolone DOC-[b]-treated HTC cells	25.0 ± 0.9
Me_C + (spironolactone DOC)[b]-treated HTC cells	10.6 ± 1.0

[a]Desaturation of [1-^{14}C]eicosa-8,11,14-trienoic acid (100 nmol, 0.25 μCi per tube) was measured by incubating 5 mg of extracted microsomes (Me_C) with 0.6 mg per tube of cytosol from control or 11-deoxycorticosaterone (DOC)-treated HTC cells.

[b]In these experiments, after the incubation with cortexolone (10^{-4}M) or spironolactone (10^{-4}M) for 2 h, DOC was added (10^{-6}M) to the same flasks and the cells were incubated with the two hormones for another 12 h. Cytosolic fractions were prepared as described in Materials and Methods. In all cases, the final incubation volume was 1.5 mL. The results are given as the mean of three independent determinations ± SEM.

somes with a low ionic strength solution. The depression of Sp_{DOC} disappeared after proteolysis of the factor with trypsin, unless the trypsin had been previously inactivated by trypsin inhibitor.

Table 5 provides additional data concerning the nature of the Δ5 desaturase inhibitory factor induced by DOC. The depressive effect of different soluble proteins (Sp) was tested. The proteins were extracted from liver microsomes of control or DOC-treated rats, segregated by ultrafiltration according to their molecular weights (> or < than 25 kDa), and their effects on the conversion of eicosatrienoic acid to arachidonic acid were tested. The Sp fraction containing protein with MW higher than 25 kDa (H-Sp) restored the Δ5 desaturation capacity of extracted microsomes. This effect was observed not only with the H-Sp fraction from control livers, but also with the same fraction of DOC-treated rats. By comparison, the addition of the L-Sp fraction (MW < 25 kDa) from control rats to extracted microsomes did not re-establish the enzymatic activity lost after washing the microsomes. The L-fraction from DOC-treated rats inhibited rather than returned Δ5 desaturase activity to values obtained with crude microsomes (M_C) (Table 5).

HTC cells were treated with cortexolone or spironolactone to determine which steroid receptors were involved in the mechanism by which the protein factor inhibits Δ5 desaturase activity. As can be seen in Table 6, when HTC cells were pre-treated with the antiglucocorticoid cortexolone, the inhibitory action of DOC on Δ5 desaturase ac-

tivity was abolished and the values obtained in the conversion of eicosatrienoic acid to arachidonic acid were similar to those obtained with the addition of control cytosol to Me_C. By contrast, pre-treatment of HTC cells with the antimineralocorticoid spironolactone did not modify the inhibitory action of DOC on Δ5 desaturase activity, and the percent conversion obtained was similar to that obtained when cytosol from DOC-treated HTC cells was added directly to Me_C.

DISCUSSION

Previous biochemical studies of dexamethasone action in the biosynthesis of arachidonic acid in liver have emphasized the possible role of hormone-induced gene activation. This activation also produced an enhancement in the synthesis of specific proteins (1,7) that lead to an inhibition in Δ5 and Δ6 desaturase enzymes and, at the same time, to an increase in Δ9 desaturase. Another steroid hormone, 11-deoxycorticosterone, is able to induce a regulatory protein factor that modulates Δ9 desaturating reactions in hepatoma tissue culture cells (HTC) (Marra, C.A., and M.J.T. de Alaniz, unpublished data). Recent studies on aldosterone have, in general, emphasized its action as an active transepithelial sodium carrier (21–23). Our study provides the first evidence that aldosterone affects the biosynthesis of arachidonic acid in liver.

Although treatment of rats with dexamethasone (3), DOC or ALDO decreased Δ5 desaturase activity in liver (Table 1), the mechanism of action for these hormones is not the same. DOC is able to synthesize a soluble inhibitory factor that is loosely bound to microsomes (3). In the present study, this factor also was found in a soluble fraction obtained after washing crude microsomes from DOC-treated rats, with a low ionic strength solution (Table 2).

The results in Table 1 suggest that a protein factor participates in the regulatory effect of DOC on the conversion of eicosatrienoic acid to arachidonic acid. Isolated cells exposed to dexamethasone or DOC produced this regulatory protein, while trypsin digestion impaired its biological activity. The hormonally-induced factor present in the cytosolic fraction of treated cells strongly depressed liver microsomal Δ5 desaturase activity (Table 3). The production of this inhibitory agent by isolated cells can be detected *in vitro*, where the interference of many complex physiological interaction does not occur as it does when the hormonal effect was tested in the whole animal.

Experiments have begun in an attempt to isolate a soluble factor responsible for the inhibition of the conversion of eicosatrienoic acid to arachidonic acid by separating the factor into light and heavy fractions. Table 5 shows that the inhibitory activity of Δ5 desaturase enzyme took place in a soluble fraction containing protein of MW lower than 25 kDa (L-Sp). However, further research is needed to characterize this protein.

The effect of aldosterone on the conversion of eicosatrienoic acid to arachidonic acid was different from those of DOC and dexamethasone. The former seems not to be mediated by a protein soluble factor induced by the hormone (Table 2), because the recovery of Δ5 desaturase activity for Me_C or Me_{ALDO} obtained by addition of soluble protein or liver cytosol from ALDO-treated rats was similar to that obtained by addition of the same fractions from control rats.

The treatment of rats with aldosterone significantly depressed Δ5 desaturase activity in both crude and extracted liver microsomes. The mechanisms by which ALDO exerts its effect on arachidonic acid biosynthesis seems to be different from those evoked by DOC (Table 2) and dexamethasone (3). The effect of aldosterone on Δ5 desaturase activity would be the consequence of the modification of the microsomal membrane properties which would take further action on the Δ5 desaturase activity. No protein messenger, induced by the hormone, would be involved in this mechanism. A similar mechanism of action was proposed previously to explain the regulatory effect displayed by testosterone on desaturase activities (24). We have already reported that the treatment of rats for 24 h with aldosterone significantly alters the fatty acid composition of liver microsomal membranes (25). The most important changes observed were a decrease in the percent distribution of palmitic, stearic, arachidonic, docosapentaenoic and docosahexaenoic acids, with a concomitant increase in the fractional composition of palmitoleic, oleic and linoleic acids. These modifications were reflected in a significant increase in the monoenoic/saturated fatty acid ratio and a decrease in the arachidonic/linoleic ratio. Since aldosterone has been found to be able to modify the fatty acid composition of liver microsomal membranes (25), it may be suggested that its inhibitory effect on Δ5 desaturase would be the direct consequence of such changes. However, whether the dexamethasone- or DOC-induced inhibition of Δ5 desaturase enzyme is mediated through glucocorticoid receptor occupancy is not known. DOC may, indeed, function as a glucocorticoid, inducing certain enzyme activities in HTC cells (11), and it is known that it is more effective than other steroids in displacing glucocorticoids from their receptors (26,27). We therefore suggest that the diminished conversion of eicosatrienoic acid to arachidonic acid evoked by DOC may be due to the production of a counteracting protein mediator released or activated in response to hormonal action through glucocorticoid receptor occupancy. In the present study we have demonstrated that glucocorticoid receptor structures are involved in the depression of Δ5 desaturase activity by DOC, because the inhibition of eicosatrienoic acid desaturation provoked by this hormone was blocked by pre-treatment of HTC cells with the antiglucocorticoid cortexolone (Table 6).

It is generally accepted that spironolactone is able to displace mineralocorticoid hormones from their receptor sites, and thereby antagonize hormone action (13,28). In our study using spironolactone (Table 6), mineralocorticoid receptors were not involved in the mechanism by which aldosterone or DOC depressed Δ5 desaturase activity.

In conclusion, the data presented here provide good evidence that DOC, which exerts physiologic and metabolic effects different from those of dexamethasone, can display similar mechanisms of action for the inhibition of arachidonic biosynthesis. We postulate that this inhibitory response is mediated by a soluble protein with a MW lower than 25 kDa. The induction of this regulatory factor requires glucocorticoid receptor occupancy and protein synthesis. Moreover, our results show that deoxycorticosterone and aldosterone depress Δ5 desaturase activity by different mechanisms of action.

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Effects of Various Dietary Fats on Cardiolipin Acyl Composition During Ontogeny of Mice[1]

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Cardiolipin (CL) is a unique mitochondrial phospholipid, containing up to 85 wt% 18:2n-6 in mammals. The influence of maternal dietary fatty acids on the acyl composition of offspring CL has not been examined previously. Adult female mice were thus fed diets rich in 18:1n-9 (olive oil), 18:2n-6 (safflower oil), 18:3n-3 (linseed oil) or 20:5n-3 and 22:6n-3 (fish oil/safflower, 9:1, w/w), for a five month period, encompassing two breeding cycles. Offspring from the second breeding cycle were then fed these diets. The acyl composition of CL, phosphatidylcholine and phosphatidylethanolamine from liver and heart was evaluated from mice killed 3, 18 and 42 days after parturition. The primary nutrient sources at these three time points were transplacental nutrients, breast milk and the diet, respectively. Maternal diet was found to influence the acyl composition of CL *via* both placental transfer of fatty acids and breast milk. Fish oil feeding resulted in replacement of a substantial portion of 18:2n-6 with 22:6n-3; after 42 days, the area% of 18:2n-6 in heart CL was reduced from 62% in safflower oil fed mice to 12%. In comparison to fish oil feeding, linseed oil feeding resulted in a much lower accumulation of 22:6n-3. Olive oil feeding resulted in substantial replacement of 18:2n-6 with 18:1n-9 (18:2n-6 was reduced from 62% to 31%). Physiologically, these findings are relevant because changes in CL acyl composition may influence the activity of associated inner mitochondrial membrane enzymes.

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Cardiolipin (CL) [1′-(1,2-diacyl-*sn*-glycero-3-phosphoryl)-3′-(1″,2″-diacyl-*sn*-glycero-3″-phosphoryl)-*sn*-glycerol; or 1,3-*bis*(3-*sn*-phosphatidyl)glycerol] is a unique mitochondrial phospholipid. In mammals, CL may contain up to 85 wt% 18:2n-6 (1–3). CL is known to be associated with several inner mitochondrial membrane proteins (4–9), most notably cytochrome *c* oxidase (10–12). The four fatty acid chains of CL may exert a variety of biophysical effects. There is evidence from both feeding studies (13–15) and *in vitro* studies with CL derivatives (2,10,11,16–18) that modulation of the acyl chain of CL can influence the activity of associated inner mitochondrial membrane proteins. We have demonstrated that alteration of the acyl moiety of CL can influence the binding to antiphospholipid antibodies (aPL) *in vitro* (19). These effects of acyl modification of CL are nutritionally relevant since the acyl moiety of CL can be altered by diet (13,15,20–24).

The influences of long-term maternal fat consumption on the CL acyl composition of offspring tissues during ontogeny has not been studied previously. We were specifically interested in the extent to which dietary fatty acids of the n-9 and n-3 series could displace the predominating n-6 fatty acid, 18:2n-6, from CL.

MATERIALS AND METHODS

Experimental animals. The initial experimental animals were four-week-old, murine-pathogen-free, female and male Swiss Webster mice, weighing 25–30 g initially (Bantin and Kingman, Fremont, CA). There were nine female mice per treatment housed in groups of five and four in 27 × 17 × 13 cm clear plastic cages with bedding. Males were fed a non-purified diet (Rodent Laboratory Chow® #5001, Purina Mills, St. Louis, MO) and were housed in groups of four. The dams received the non-purified diet for two weeks, followed by the experimental diets for a five-month period which encompassed two breeding cycles, five weeks apart. First pregnancies are characterized by large variations in the number of pups born and by cannibalism. Hence, offspring from the second pregnancy were analyzed.

During mating periods, one male was added to the cage of three females for a three-day period. Two weeks later, males were re-introduced to those non-pregnant females (as determined by vaginal plugs and weight gain) in a 1:1 ratio for one week. On day 14 of gestation, pregnant mice were housed individually. This breeding scheme resulted in 5–9 successful pregnancies per treatment. Litters were randomly normalized from 10–15 pups to 10 pups on day 3 *postpartum*. On day 18, the dams were removed, and the litters were culled to 6. On day 42, the remaining pups were killed by CO_2 intoxication. Tissues were analyzed from dams, and from pups which were killed 3, 18 and 42 days after parturition. The primary nutrient sources at these three time points were transplacental nutrients, breast milk and the diet, respectively.

Experimental diets. The experimental diets consisted of fat-free AIN 76A meal diets (25) (Dyets Inc., Bethlehem, PA) modified by addition of either 10 wt% safflower oil (Dyets Inc.), linseed oil (Spectrum Marketing, Petaluma CA), olive oil (G. Sensat, extra virgin #5, Specialty Food and Beverage Sales, West Milford, NJ), or a 9:1 (w/w) mixture of vacuum deodorized menhaden oil (National Institute of Health Biomedical Test Material Lot #L89195BB, Bethesda, MD) and safflower oil (Table 1). The fat-free diet mix, safflower oil and olive oil were stored refrigerated, linseed and fish oils were stored at −70°C under N_2. To minimize oxidation, all oils contained 0.02 wt% *tert*-butylhydroquinone (Aldrich Chemical Co., Milwaukee, WI). The diets were prepared in a commercial mixer in 5-kg lots, sealed under N_2 in freezer bags in 250-g quantities, and stored at −70°C.

Mice received *ad libitum* amounts of fresh feed in ceramic feed cups each evening in a humidity-controlled room with a dark cycle from 1800–0700 hours. Uneaten food was discarded each evening. Deionized water was provided *via* a pressure sensitive nozzle. Feed cups,

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Abbreviations: ANOVA, analysis of variance; aPL, antiphospholipid antibodies; CL, cardiolipin; FAME, fatty acid methyl ester; HPTLC, high-performance thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

TABLE 1

Fatty Acid Composition of the Experimental Diets[a]

Fatty acid	Safflower oil	Olive oil	Linseed oil	Fish oil/safflower oil (9:1, w/w)[b]
14:0	0.2	0.0	0.0	6.4
16:0	7.2	10.7	5.0	16.0
16:1n-7	0.0	0.8	0.0	7.8
18:0	2.2	3.6	4.2	2.7
18:1n-9	12.0	78.2	16.3	9.7
18:2n-6	77.7	5.3	16.3	9.0
18:3n-3	0.0	0.7	58.2	1.1
20:2n-6	0.0	0.0	0.0	0.2
20:3n-3	0.0	0.0	0.0	0.1
20:5n-3	0.0	0.0	0.0	12.8
22:5n-3	0.0	0.0	0.0	2.0
22:6n-3	0.0	0.0	0.0	11.2
Other saturated FA[c]	0.7	0.7	0.0	2.1
Other monoenoic FA[d]	0.0	0.0	0.0	7.1
Other dienoic FA[e]	0.0	0.0	0.0	2.0
Other trienoic, tetraenoic and pentaenoic FA[f]	0.0	0.0	0.0	9.8

[a]Values are expressed as area percent and represent the mean of two determinations.
[b]Source: NIH Fish Oil Test Material Program QA/QC Report, 1989.
[c]12:0, 13:0, 15:0, 17:0, 20:0, 22:0, 15:0 *iso*.
[d]14:1n-5+7 (14:1n-5 plus 14:1n-7), 16:1n-5+9+11, 18:1n-7, 20:1n-5+7+9+11+13, 22:1n-7+9+11+13, 24:1n-9.
[e]16:2n-4+6+7, 18:2n-4+5+9.
[f]16:3n-3+4, 16:4n-1, 18:3n-4+6, 18:4n-1+3, 20:4n-3+6, 21:5n-3, 22:4n-6, 22:5n-6.

cages and bedding were cleaned every 2–4 d as needed.

Lipid analysis. Tissue lipids were extracted (26), separated by high-performance thin-layer chromatography (HPTLC) (27) and then methylated (28). Fatty acid methyl esters (FAME) were resolved by gas chromatography and detected with a flame ionization detector as previously described (21), except for the modifications listed below.

To analyze day 42 livers by HPTLC, it was necessary to first remove neutral lipids. Lipid extracts from 300 mg of liver were dissolved in 2 mL of hexane, and applied to pre-washed 100 mg silica columns equipped with stainless steel frits and 10-mL reservoirs (Analytichem International, Harbor City, CA). Columns were mounted to a Supelco Visiprep™ Solid Phase Extraction Vacuum Manifold (Bellefonte, PA). Neutral lipids were eluted with 12 mL of $CHCl_3$. CL, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine (PE) were eluted with 4 mL of $CHCl_3/CH_3OH$ (2:1, vol/vol), and phosphatidylcholine (PC) and sphingomyelin were eluted with 4 mL CH_3OH.

For gas chromatography, FAME were injected with a Hewlett-Packard (HP) model 7673 autosampler and resolved with an HP gas chromatograph Model 5890A equipped with a DB-23 capillary column (25 m × 0.25 μm i.d., 50% cyanopropyl phase, 0.25 μm film thickness; J&W Scientific, Folsom, CA). Integrator data were electronically transferred to a Macintosh™ computer, where the data were automatically sorted based on the retention time of standard FAME (NuChek Prep, Elysian, MN), and then converted to area percentages in Microsoft Excel® (Redmond, WA).

Statistics. Results are expressed as area% and were evaluated with analysis of variance (ANOVA) (29) using the statistical package Super ANOVA (Abacus Concepts, Berkeley, CA).

RESULTS

Gestational length, litter size, survivorship and growth parameters. Diet did not have a significant effect on the gestational period, the litter size or the average weight of offspring killed on day three after adjusting for litter size ($P > 0.44$). Interestingly, the number of "successful pregnancies" (out of nine dams per treatment) were: Nine, fish oil; seven, linseed; six, safflower; and five, olive. A pregnancy was considered successful if all of the following criteria were met: i) The dam became pregnant after three exposures to a male; ii) at least six healthy pups were born; and iii) after culling to 10 pups on day three, at least six healthy offspring survived to weaning on day 18. Non-successful pregnancies were characterized by still births, maternal abandonment, cannibalism and large variation in pup size within the litter.

Safflower oil fed dams were found to gain weight at a slightly faster rate than dams from all other groups (analysis of the residuals of a third-order polynomial regression equation; $P < 0.05$). There were also small, but statistically significant, differences in the body weights of the offspring for the various treatments ($P < 0.01$; ANOVA with litter as a nested factor). Body weights ranged from 8.2–10.6 g at 18 d, and from 30.1–33.5 g at 42 d for the various dietary treatments. Dietary fat intake had no significant effect on the relative organ weights (organ weight/body weight) of liver and kidney of day 18 mice, but did elicit small but significant effects ($P < 0.05$) on relative brain, spleen and thymus weights. These data are discussed in detail elsewhere (Berger, A., German, J.B., Keen, C., Ansari, A., and Gershwin, M.E., manuscript submitted for publication).

Influence of age and diet on acyl modification of hepatic and cardiac CL. The CL acyl composition of the pups was

TABLE 2

Fatty Acid Composition of Cardiac Cardiolipin from Mice Killed 3, 18 and 42 Days After Parturition[a]

	Day 3[b]				Day 18				Day 42			
Fatty acid	Saf	Oli	Lin	Fish	Saf	Oli	Lin	Fish	Saf	Oli	Lin	Fish
16:0	2.8^{d}	8.7^{d,e}	20.3^{e}	9.0^{d,e}	2.2	3.3	3.8	2.4	4.7^{e}	2.5^{d,e}	0.0^{d}	3.2^{d,e}
18:0	3.6	3.4	13.7	8.7	1.8^{d,e}	2.6^{e}	1.6^{d,e}	0.8^{d}	2.9	1.0	0.3	1.5
18:1n-9+7	27.0^{d}	55.2^{e}	33.1^{d}	33.9^{d}	11.2^{d}	47.6^{f}	25.1^{e}	21.9^{e}	11.2^{d}	41.9^{f}	14.0^{d,e}	21.6^{e}
18:2n-6	44.7^{e}	18.7^{d}	13.2^{d}	15.7^{d}	61.6^{g}	31.2^{e}	41.6^{f}	12.1^{d}	66.7^{g}	38.3^{e}	50.7^{f}	19.0^{d}
20:2n-6	3.6^{e}	0.4^{d}	0.0^{d}	1.0^{d,e}	14.0^{f}	1.7^{d,e}	2.9^{e}	0.4^{d}	4.0^{e}	0.7^{d}	0.0^{d}	0.3^{d}
20:3n-6	2.1	1.9	0.0	3.5	3.3^{d}	5.3^{e}	3.0^{d}	4.2^{d,e}	2.0^{d,e}	6.2^{f}	4.0^{e,f}	0.5^{d}
20:4n-6	8.8	3.4	10.8	4.8	2.2	2.4	1.0	2.1	2.7^{e}	2.3^{e}	1.3^{d,e}	0.4^{d}
22:4n-6	2.1^{f}	0.0^{d}	0.0^{d}	0.5^{e}	0.9^{d,e}	0.9^{d,e}	1.5^{e}	0.0	0.0	0.0	0.0	0.0^{d}
22:5n-6	1.6^{e}	0.0^{d}	0.0^{d}	0.0^{d}	1.4^{f}	0.7^{e}	0.0^{d}	0.0^{d}	4.1^{e}	1.4^{d}	0.0^{d}	0.0^{d}
18:3n-3	0.0	0.0	0.0	0.0	0.0^{d}	0.0^{d}	4.1^{e}	0.0^{d}	0.0^{d}	0.2^{d}	6.6^{e}	0.1^{d}
20:3n-3	0.0	0.0	0.0	0.6	0.01^{d}	0.0^{d}	7.6^{e}	0.0^{d}	0.0^{d}	0.0^{d}	1.5^{e}	0.0^{d}
20:5n-3	0.1^{d}	0.0^{d}	0.0^{d}	2.5^{e}	0.0	0.0	0.4	0.4	0.0	0.0	0.0	0.4
22:5n-3	0.0^{d}	0.5^{d,e}	0.0^{d,e}	3.7^{e}	0.0^{d}	0.3^{d}	2.3^{e}	5.6^{f}	0.0^{d}	0.1^{d}	5.8^{e}	1.1^{d}
22:6n-3	0.6^{d}	0.0^{d}	3.6^{e}	12.0^{f}	0.0^{d}	1.1^{d}	2.1^{d}	48.4^{e}	0.0^{d}	2.5^{d}	15.8^{e}	48.3^{f}
Others[c]	3.0	7.8	5.3	4.1	1.4	2.9	3.0	1.7	1.7	2.9	0.0	3.6

[a]Abbreviations are as follows: Saf, safflower oil fed group; Oli, olive oil fed group; Lin, linseed oil fed group; and Fish, fish oil fed group. Results are expressed as the mean area percent of each fatty acid in CL. 18:1n-9+7 Denotes the sum of 18:1n-9 and 18:1n-7. These isomers were combined since their chromatographic separation was not reliable. When 18:1n-9 was considered alone in the analyses, the statistical trends were identical. Generally, there were highly significant differences ($P < 0.0001$) between the time points and between organs. For simplicity, Tables include one-way ANOVA evaluation of the diet effects at each time point.

[b]Due to limited tissue availability on day 3, it was necessary to pool day 3 hearts from several mice. This resulted in an n of 1 for fish and linseed, 2 for safflower and 5 for olive oil. Statistical significance was thus achieved only when differences were quantitatively large. n Ranged from 3-6 for days 18-42.

[c]Includes 14:0, 14:1n-5, 16:1n-7, 18:3n-6, 20:0, 22:0, 24:0, 20:1n-9, 22:1n-9 and 24:1n-9.

[d,e,f,g]Values within each time point sharing a superscript or lacking a superscript are not significantly different at $P > 0.05$ (Fisher's protected least significant difference). No statistics were performed on the combined fatty acids in the "Others" category.

found to depend on the age of the pups, the diet consumed by both the dams and the pups and the tissue examined.

Age dependency was evident in all dietary groups (Tables 2 and 3). Levels of 18:1 (18:1n-9 plus 18:1n-7) and 20:4n-6 (though only in the heart) were higher at birth and these acids were increasingly replaced with other fatty acids (most notably 18:2n-6) through day 18 and 42 (Tables 2 and 3).

There was also clear evidence that maternal diet influenced the acyl composition of CL in newborn (day 3) and in nursing (day 18) pups. In liver and heart CL, mice fed the n-6 rich safflower oil had higher levels of 18:2n-6 and its metabolites 22:5n-6 and 20:2n-6 at birth, and increasingly higher levels at each respective time point. Mice fed olive oil had higher levels of 18:1. Mice fed linseed oil had higher levels of 18:3n-3 and its metabolites 22:6n-3 and 20:3n-3 (except cardiac CL from day 3 mice). Mice fed fish oil had higher levels of 22:6n-3. Relative to safflower oil feeding, the feeding of all other diets resulted in strikingly lower levels of 18:2n-6. The accumulation of the elongation products 20:2n-6 and 20:3n-3 declined between days 18 and 42 (Tables 2 and 3).

The acyl composition of heart CL was extremely sensitive to the difference between feeding of 18:3n-3 (linseed oil) and 20:5n-3 and 22:6n-3 (fish oil). As compared to mice fed fish oil, mice fed linseed oil for 42 d accumulated much less 22:6n-3 (48% *vs.* 16%, respectively, in the heart) but, interestingly, accumulated more 22:5n-3.

The accumulation of 22:6n-3 in CL was also tissue specific. In comparison to the heart, the liver accumulated less 22:6n-3 at all time points following fish oil feeding and there was a similar accumulation of 22:6n-3 after 42 d feeding with both linseed oil and fish oil. Hepatic levels of 22:6n-3 were not maximized until day 42.

Comparison of the acyl composition of CL, PC and PE. Historically, CL has been considered to elicit a high degree of acyl chain selectivity due to the predominance of 18:2n-6 (1). It was therefore interesting to compare the acyl composition of CL as a function of age and diet to PC and PE, which are thought to accumulate dietary fatty acids with less discrimination. A similar pattern of incorporation of the various dietary fatty acids into CL, PC and PE from the two organs was evident at day 18, however, there were some remarkable differences (Tables 4 and 5). Following feeding of the various n-9, n-6 and n-3 fatty acids, 18:1 and 18:2n-6 were generally present at lower levels in PC and PE, whereas 20:4n-6, 22:5n-6, 20:5n-3 and 22:5n-3 were higher, relative to CL. Hepatic levels of 22:6n-3 were higher in PC and PE than CL on day 18. The dramatically higher level of 22:5n-3 in cardiac PC and PE observed with linseed oil feeding relative to fish oil feeding was not apparent in CL. It is also notable that in cardiac PE, the summed area% of 20:5n-3, 22:5n-3 and 22:6n-3 were equivalent after linseed and fish oil feeding. In PE, 22:6n-3 largely replaced 20:4n-6 and 22:5n-6, whereas 22:6n-3 replaced 18:2n-6 in CL.

Selectivity of incorporation of various fatty acids. Since the experimental diets contained differing amounts of 18:1n-9, 18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3, in order to assess the selectivity for incorporation of these dietary fatty acids into the CL pool, a variable termed the "relative selectivity index" was developed (Table 6). The precursor fatty acids were considered to be 18:1n-9 (olive oil), 18:2n-6 (safflower oil), 18:3n-3 (linseed oil) and the summed quantity of 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3

TABLE 3

Fatty Acid Composition of Hepatic Cardiolipin from Mice Killed 3, 18 and 42 Days After Parturition[a]

	Day 3[b]				Day 18				Day 42			
Fatty acid	Saf	Oli	Lin	Fish	Saf	Oli	Lin	Fish	Saf	Oli	Lin	Fish
16:0	6.7^{e}	0.0^{d}	13.3^{e}	14.2^{e}	3.1^{d}	5.6^{d}	12.7^{e}	5.4^{d}	3.0^{d}	14.4^{e}	8.9^{e}	22.0^{f}
18:0	6.8^{d,e}	0.0^{d}	5.8^{d,e}	11.9^{e}	2.9^{d}	2.7^{d}	8.3^{e}	3.2^{d}	3.7^{d}	11.8^{e}	11.4^{e}	18.9^{f}
18:1n-9+7	16.0^{d}	51.9^{f}	26.1^{e}	25.8^{e}	5.7^{d}	31.6^{g}	15.5^{e}	21.2^{f}	18.6^{d,e}	37.3^{e}	16.8^{d}	14.6^{d}
18:2n-6	56.1^{e}	35.9^{d}	40.0^{d,e}	30.6^{d}	72.5^{f}	52.8^{d,e}	46.6^{d}	61.1^{e}	66.0^{e}	19.8^{d}	33.4^{e}	18.0^{d}
20:2n-6	4.7^{e}	0.0^{d}	0.9^{d}	0.3^{d}	8.9^{e}	0.4^{d}	0.4^{d}	0.7^{d}	2.2^{d}	0.0^{d}	0.2^{d}	0.1^{d}
20:3n-6	0.9^{d}	0.0^{d}	0.2^{d}	1.1^{e}	0.1^{d}	2.0^{e}	0.4^{d}	1.2^{d,e}	0.9^{d}	2.6^{e}	1.1^{d}	0.8^{d}
20:4n-6	2.5	0.0	0.7	1.0	2.8^{e}	1.2^{d,e}	0.7^{d}	0.4^{d}	1.4^{d}	3.7^{e}	1.5^{d}	2.4^{d,e}
22:4n-6	1.1	0.0	0.0	0.7	0.5	1.5	0.0	0.0	0.0^{d}	0.0^{d}	0.0^{d}	0.0^{d}
22:5n-6	2.1	0.0	0.9	0.0	2.7^{e}	0.2^{d}	0.0^{d}	0.0^{d}	0.9^{d}	2.2^{e}	0.0^{d}	0.0^{d}
18:3n-3	0.0	0.0	2.2	0.0	0.0^{d}	0.0^{d}	5.6^{e}	0.1^{d}	0.0^{d}	0.1^{d}	5.9^{e}	0.2^{d}
20:3n-3	0.0	0.0	0.6	1.5	0.0^{d}	0.4^{d}	6.5^{e}	0.2^{d}	0.0^{d}	0.0^{d}	2.6^{e}	0.0^{d}
20:5n-3	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.2	0.0^{d}	0.0^{d}	3.6^{e}	4.2^{e}
22:5n-3	0.0	0.0	1.5	0.4	0.0	0.0	0.0	0.3	0.0^{d}	0.0^{d}	1.1^{e}	0.9^{e}
22:6n-3	0.0^{d}	0.0^{d}	1.1^{d,e}	5.0^{e}	0.3^{d}	0.2^{d}	0.8^{d,e}	2.2^{e}	0.0^{d}	3.4^{d}	10.9^{e}	13.6^{e}
Others[c]	3.1	12.2	6.7	6.8	0.5	1.4	2.5	3.8	3.3	4.7	2.6	4.3

[a,c,d,e,f,g]See corresponding footnotes in Table 2. In olive oil fed mice killed on day 3, the major fatty acid in the "Others" category was 16:1n-7.
[b]n = 2. For olive oil and linseed oil fed groups, n = 3 for safflower oil and fish oil fed groups. n Ranged from 3-6 on days 18-42.

TABLE 4

Fatty Acid Composition of Hepatic and Cardiac Phosphatidylcholine from Mice Killed 18 Days After Parturition[a]

	Heart[b]				Liver			
Fatty acid	Saf	Oli	Lin	Fish	Saf	Oli	Lin	Fish
16:0	20.5	21.6	21.0	26.4	31.0^{d}	33.4^{d,e}	31.4^{d}	36.2^{e}
18:0	25.6^{e}	25.4^{e}	21.6^{d}	20.7^{d}	12.7	11.0	12.9	11.2
18:1n-9+7	8.0^{d}	23.4^{e}	10.1^{d}	6.4^{d}	6.0^{d}	27.8^{e}	10.7^{d}	8.4^{d}
18:2n-6	9.2^{f}	3.6^{d}	5.8^{e}	2.6^{d}	20.0^{f}	5.9^{d}	14.4^{e}	6.4^{d}
20:2n-6	1.4	2.4	0.6	0.0	1.2^{e}	4.0^{f}	0.1^{d}	0.0^{d}
20:3n-6	0.7	1.4	1.0	0.0	1.3^{d,e}	1.9^{e}	1.5^{e}	0.7^{d}
20:4n-6	17.2^{e}	10.2^{d,e}	4.1^{d}	2.4^{d}	18.3^{f}	9.4^{e}	3.0^{d}	3.4^{d}
22:4n-6	7.1^{f}	2.4^{e}	0.0^{d}	0.0^{d}	0.3	0.0	0.0	0.0
22:5n-6	9.8^{f}	2.6^{e}	0.0^{d}	0.0^{d}	8.3^{f}	1.8^{e}	0.0^{d}	0.0^{d}
18:3n-3	0.0^{d}	0.0^{d}	0.8^{e}	0.0^{d}	0.0^{d}	0.0^{d}	1.5^{e}	0.0^{d}
20:3n-3	0.0^{d}	0.0^{d}	1.2^{e}	0.0^{d}	0.0^{d}	0.0^{d}	0.7^{e}	0.0^{d}
20:5n-3	0.0^{d}	0.0^{d}	1.5^{e}	0.6^{d}	0.0^{d}	0.0^{d}	7.7^{f}	2.8^{e}
22:5n-3	0.0^{d}	1.8^{e}	17.7^{g}	5.6^{f}	0.0^{d}	0.0^{d}	1.7^{e}	1.6^{e}
22:6n-3	0.5^{d}	4.2^{e}	14.4^{f}	35.3^{g}	0.8^{d}	4.2^{d}	13.9^{e}	28.7^{f}
Others[c]	0.0	1.0	0.2	0.0	0.1	0.6	0.5	0.6

[a,c,d,e,f,g]See corresponding footnotes in Table 2.
[b]n Ranged from 3-6 for the heart and liver.

(fish oil). After 42 days, 22:6n-3 derived from precursors in fish oil (as opposed to 18:3n-3) was incorporated into cardiac CL with twice the selectivity of 18:2n-6, whereas 18:1n-9 showed less selectivity for incorporation than 18:2n-6. Conversely, in the liver, there was twice the selectivity for incorporation of 18:2n-6 than 22:6n-3.

DISCUSSION

Age and diet related replacement of monoenoic acids with linoleate. Independent of diet, all the experimental groups were born with a higher content of 18:1 in CL that was gradually replaced with other fatty acids derived from breast milk and the diet. The observed decreases in 18:1 and 16:1n-7, and increases in 18:2n-6 from birth through the post-weaning period have been reported in rats (30–35) and in humans (36), and may result from decreased endogenous fetal synthesis of these monoenes from acetate with age, along with increased supply of 18:2n-6 (and other maternal fatty acids) *via* placental transfer, breast milk and the diet (present study and ref. 34). In contrast to our results, rabbit heart mitochondria examined three days postnatally recently were reported to have a higher content of 18:2n-6 (92%) in CL than adult heart mitochondria (84%) (37).

Accumulation of 22:6n-3 from fish and linseed oil feeding. The substantial accumulation of 22:6n-3 in CL following fish oil feeding has been reported in some studies (13,38,39), but not in others (23,40). Factors that may account for this discrepancy are differences in the species and age of the experimental animals; the tissues examined (1); the amount of food consumed; the feeding duration (13,23); the levels of n-3 polyenoic acids in the diet (39); and the amount of competing n-9 and n-6 dietary fat-

TABLE 5

Fatty Acid Composition of Hepatic and Cardiac Phosphatidylethanolamine from Mice Killed 18 Days After Parturition[a]

	Heart[b]				Liver			
Fatty acid	Saf	Oli	Lin	Fish	Saf	Oli	Lin	Fish
16:0	8.3[d]	7.3[d]	8.2[d]	12.3[e]	14.8[d]	14.7[d]	16.2[d]	20.1[e]
18:0	27.9	29.1	27.2	30.9	27.7	30.9	28.8	27.4
18:1n-9+7	2.7[d,e]	8.1[f]	5.3[e]	1.8[d]	2.7[e]	5.4[f]	2.7[e]	1.3[d]
18:2n-6	2.6[f]	1.1[d,e]	1.1[d,e]	0.0[d]	6.3[f]	2.1[d,e]	3.2[e]	1.3[d]
20:2n-6	0.0[d]	0.9[e]	0.0[d]	0.0[d]	0.4[d]	1.8[e]	0.0[d]	0.0[d]
20:3n-6	0.0	0.6	0.1	0.0	0.9[f]	0.9[f]	0.6[e]	0.2[d]
20:4n-6	17.2[f]	25.0[g]	6.5[e]	1.8[d]	27.1[f]	28.7[g]	6.5[e]	4.8[d]
22:4n-6	8.1[f]	4.0[e]	0.1[d]	0.0[d]	2.7[f]	0.9[e]	0.0[d]	0.0[d]
22:5n-6	21.4[f]	7.1[e]	0.0[d]	0.0[d]	15.2[f]	4.2[e]	0.0[d]	0.0[d]
18:3n-3	0.0	0.0	0.1	0.0	0.0[d]	0.0[d]	0.6[e]	0.0[d]
20:3n-3	0.0[d]	0.0[d]	0.1[e]	0.0[d]	0.0[d]	0.0[d]	0.1[e]	0.0[d]
20:5n-3	2.0[d,e]	0.0[d]	2.5[e]	0.9[d,e]	0.0[d]	0.0[d]	11.0[f]	4.6[e]
22:5n-3	0.8[d]	2.1[d]	14.7[e]	1.6[d]	0.0[d]	0.2[d]	3.9[f]	2.3[e]
22:6n-3	9.0[d]	14.5[d]	33.0[e]	50.7[f]	2.2[d]	9.9[e]	26.4[f]	38.0[g]
Others[c]	0.0	0.2	1.1	0.0	0.0	0.3	0.0	0.0

[a,c,d,e,f,g]See corresponding footnotes in Table 2.
[b]n Ranged from 3–6 for the heart and liver.

ty acids (41). Another factor is that CL could be contaminated with free fatty acids during its chromatographic separation. The very high levels of 22:6n-3 incorporated into cardiac CL in the present study reflects the long-term feeding of a diet rich in n-3 polyenoic acids, but is not dependent on the fact the diet was fed through two generations, since similarly high levels of 22:6n-3 were observed in the CL of adult mice fed fish oil for five months (data not shown). The observed reductions in 18:2n-6 with fish oil feeding were not entirely due to diminished supply of 18:2n-6 in the diet. Our selectivity data, along with the demonstration that fish oils can decrease the content of 18:2n-6 in CL to a greater extent than essential fatty acid deficient diets or hydrogenated corn oil diets (15), suggests that 22:6n-3 may actively compete with 18:2n-6 for incorporation into CL.

The greater accumulation of 22:6n-3 in CL from the heart than the liver is an indication that there was selective uptake or retention by the heart since isolated heart myocytes cannot elongate 20:5n-3 or desaturate 22:5n-3 (42). Cardiac binding proteins may have high selectivity for 22:6n-3, accounting for rapid uptake of this fatty acid into heart cells (43). Decreased retroconversion (44), high selectivity for acyltransferases (45), decreased phospholipase action (46) and diminished mitochondrial oxidation (47) may also contribute to the higher level of 22:6n-3 maintained in cardiac CL.

The apparent selective uptake of 22:6n-3 into heart CL was evident during the transfer of both maternal transplacental and breast milk derived 22:6n-3. As compared to the heart (in linseed oil fed mice) there were higher levels of 18:3n-3 in CL from the liver of day three pups, and lower levels of 22:6n-3. This is an indication that there was selective uptake of placental 22:6n-3 in the heart. In fish oil fed mice, levels of 22:6n-3 were maximized by day 18 in the heart, but not until day 42 in the liver. This suggests that 22:6n-3 derived from breast milk, or released from other organs such as adipose tissue and liver, was selectively retained by heart cells. These results emphasize the importance of evaluating the phospholipid acyl moiety of more than one tissue in dietary lipid studies.

The lesser accumulation of 22:6n-3 in cardiac CL with linseed oil feeding relative to fish oil feeding (23,24) suggests that one or more steps in the conversion of 18:3n-3 to 22:6n-3 are regulated, or at least limiting. Recent evidence suggests that 20:5n-3 may be metabolized to 22:6n-3 *via* the following pathway: 5,8,11,14,17-20:5 → 7,10,13,16,19-22:5 → 9,12,15,18,21-24:5 → 6,9,12,15,18,21-24:6 → 4,7,13,16,19-22:6 (48). In short-term feeding experiments, the quantitative differences in 22:6n-3 incorporation into CL with linseed and fish oil feeding are less obvious (23). This suggests that longitudinal feeding studies need to be conducted with graded amount of n-3 fatty acids provided.

In cardiac PE, the fact that levels of 22:5n-3 were considerably higher after linseed oil feeding as compared to fish oil feeding suggests that the conversion of 22:5n-3 to 24:5n-3 may be limiting. There was no evidence from our gas chromatographic analyses that 24:5n-3 or 24:6n-3 accumulated.

Accumulation of 18:1n-9, 18:3n-3, 20:3n-3 and 20:2n-6. The extensive incorporation of 18:1n-9 into CL has been reported in rats fed 20 wt% olive oil (31) or triolein (18) for 4–5 months, but not in shorter-duration experiments (23). Oleate also has been found to increase in CL after feeding rapeseed oil and essential fatty acid deficient or low linoleic acid diets (3).

The minor accumulation of 18:3n-3 (21,49) and 20:3n-3 (24) in CL after feeding linolenate sources has been reported previously. The fact that 20:2n-6 (following safflower oil feeding) and 20:3n-3 (following linseed oil feeding) accumulated in day 18, but not day 42, mice suggests that these fatty acids were either more available during breast feeding (day 18) than during dietary ingestion (day 42); or that the Δ6 desaturase may not have been fully active in day 18 mice. There is some evidence from *in vitro* studies that "dead end" elongation products may accumulate under conditions when the desaturase system is not active (50–52).

TABLE 6

Meta-Analysis of the Selectivity of Dietary Fatty Acids for Incorporation into Cardiolipin

Organ	Species	Months fed diet	Lipid source	FA precursor	FA ratio[a]		(Reference)
Heart	Rat	1.00	Mixture	18:1n-9	0.81	δ	(23)
Heart	Rat	4.00	Olive	18:1n-9	1.48	δ	(31)
Heart	Rat	2.50	Mixture	18:1n-9	2.97	δ	(22)
Heart	Mouse	1.50	Olive	18:1n-9	3.98	δ	Present study, day 42
Heart	Rat	1.50	Rapeseed	22:1n-9	0.20	φ	(59)
Heart	Rat	1.00	Rapeseed	22:1n-9	0.43	φ	(58)
Heart	Rat	1.00	Rapeseed	22:1n-9	0.50	φ	(58)
Heart	Rat	1.00	Rapeseed	22:1n-9	0.65	φ	(32)
Heart	Rat	2.50	Rapeseed	22:1n-9	1.19	φ	(62)
Heart	Rat	0.30	Rapeseed	22:1n-9	1.27	φ	(58)
Heart	Chick	0.80	Rapeseed	22:1n-9	1.51	φ	(60)
Heart	Chick	0.80	Sunflower	18:2n-6	4.91	θ	(60)
Heart	Rat	1.50	Corn	18:2n-6	5.92	θ	(59)
Heart	Rat	4.00	Corn	18:2n-6	6.96	θ	(31)
Heart	Rat	1.00	Corn	18:2n-6	7.09	θ	(13)
Heart	Mouse	1.50	Safflower	18:2n-6	8.58	θ	Present study, day 42
Heart	Rat	12.00	Sunflower	18:2n-6	10.38	θ	(38)
Heart	Rat	5.00	Sunflower	18:2n-6	10.72	θ	(24)
Heart	Rat	2.75	Safflower	18:2n-6	12.10	θ	(49)
Heart	Rat	0.75	Coconut	18:2n-6	25.40	θ	(39)
Heart	Mouse	0.50	Safflower	18:2n-6	27.16	θ	(21)
Heart	Rat	0.75	Coconut	18:2n-6	49.63	θ	(39)
Heart	Rat	2.75	Soybean	18:3n-3	0.00	Ω	(49)
Heart	Rat	1.00	Soybean	18:3n-3	0.94	Ω	(32)
Heart	Rat	4.00	Soybean	18:3n-3	0.95	Ω	(31)
Heart	Rat	5.00	Mixture	18:3n-3	0.96	Ω	(24)
Heart	Rat	1.00	Mixture	18:3n-3	1.87	Ω	(23)
Heart	Mouse	1.50	Linseed	18:3n-3	2.72	Ω	Present study, day 42
Heart	Mouse	0.50	Linolenate	18:3n-3	10.00	Ω	(21)
Heart	Rat	0.75	Menhaden	Σ n-3 in FO[b]	0.91	Ω	(39)
Heart	Rat	3.00	Cod Liver	Σ n-3 in FO	1.06	Ω	(40)
Heart	Rat	0.75	Menhaden	Σ n-3 in FO	1.08	Ω	(39)
Heart	Rat	0.75	Menhaden	Σ n-3 in FO	2.36	Ω	(39)
Heart	Rat	1.00	Mixture	Σ n-3 in FO	5.36	Ω	(23)
Heart	Rat	1.00	Sardine	Σ n-3 in FO	5.68	Ω	(13)
Heart	Rat	12.00	Tuna	Σ n-3 in FO	9.12	Ω	(38)
Heart	Mouse	1.50	Menhaden	Σ n-3 in FO	17.81	Ω	Present study, day 42
Liver	Rat	2.50	Mixture	18:1n-9	1.64	δ	(22)
Liver	Mouse	1.50	Olive	18:1n-9	2.67	δ	Present study, day 42
Liver	Rat	0.30	Corn	18:2n-6	6.92	θ	(13)
Liver	Rat	5.00	Sunflower	18:2n-6	8.16	θ	(24)
Liver	Mouse	1.50	Safflower	18:2n-6	8.49	θ	Present study, day 42
Liver	Rat	2.75	Safflower	18:2n-6	9.12	θ	(49)
Liver	Mouse	0.50	Safflower	18:2n-6	26.70	θ	(21)
Liver	Rat	2.75	Soybean	18:3n-3	0.00	Ω	(49)
Liver	Rat	5.00	Linseed	18:3n-3	0.99	Ω	(24)
Liver	Mouse	0.60	Linseed	18:3n-3	3.73	Ω	Present study, day 42
Liver	Mouse	0.50	Linolenate	18:3n-3	5.90	Ω	(21)
Liver	Rat	0.30	Sardine	Σ n-3 in FO	1.48	Ω	(13)
Liver	Mouse	1.50	Menhaden	Σ n-3 in FO	4.02	Ω	Present study, day 42

[a]Calculated as the ratio of the mol%, wt% or area% of 18:1n-9 (δ), 22:1n-9 (φ), 18:2n-6 (θ), or 22:6n-3 (Ω) in CL divided by the respective mol%, wt% or area% of the designated precursor fatty acid.
[b]FO, fish oil.

Mechanisms of acyl specificity in cardiolipin. Linoleate is known to be a major fatty acid in CL from a variety of mammalian tissue sources except for rat brain, lung and testes (1,53). Our data indicate that 18:2n-6 accumulates in CL because it is very abundant in most animal diets, and that other fatty acids, such as 18:1n-9 and 22:6n-3, may replace 18:2n-6 when provided in sufficient amounts in the diet. There is not a uniquely high selectivity for incorporation (Table 6) or retention of this fatty acid in CL pools. Rather, it has been shown that 18:2n-6 levels in CL change dynamically in response to dietary 18:2n-6 supply (20,22,32,54,55). Interestingly, we found that a marine shark (brown smoothhound shark, *Mustelus henlei*) caught in Tomales Bay, Northern California, had only 3 area% 18:2n-6 in heart CL, but had high levels of n-9 monoenoic acids (49% 18:1), and n-3 polyenoic acids (12% 22:5n-3, 18% 22:6n-3) (unpublished data). These data likely reflect the fact that sharks consume a diet low in 18:2n-6 and high in n-3 polyenoic acids.

The assumed high selectivity of 18:2n-6 for CL has not been demonstrated *in vitro* (1). Schlame and Rüstow (2) have provided some evidence that monolysoCL can be

enriched with 18:2n-6 when 1-palmitoyl-2-linoleoyl PC is present in the incubation system (1-palmitoyl-2-docosahexaenoyl PC was not evaluated). This observation, and the fact that CL is synthesized from inner mitochondrial membrane precursors that are not particularly rich in 18:2n-6, suggests that extensive retailoring of CL may occur (56,57).

The replacement of 18:2n-6 with 22:6n-3 and 18:1n-9 suggests that the enzymes responsible for remodeling and maintaining acyl specificity in CL (phospholipases and acyltransferases) can recognize and select for fatty acids of different unsaturation and different chain length. In other phospholipids, fatty acids that are more structurally similar may replace one another (*e.g.*, 20:5n-3 may replace 20:4n-6; and 22:6n-3 may replace 22:5n-6) (42).

There appears to be no special selectivity for incorporation of 22-carbon fatty acids (compare 22:1n-9 and 22:6n-3; Table 6). Wolff and Entressangles (3) suggest that there may be selectivity for double bond positions 8 and 11 in both 18- and 20-carbon fatty acids, based on the results of depletion studies. This selectivity is difficult to discern from dietary feeding studies. The following fatty acids are more abundant in CL relative to other phospholipids or can be enriched in CL by dietary means: 16:1n-7, 18:1n-7, 18:1n-9, 18:2n-6, 5*t*,9*c*,12*c*-octadecatrienoate, 20:2n-6, 20:3n-3, 22:1n-9 and 22:6n-3 (21,58–61). The following dietary fatty acids are incorporated into CL to a lesser extent: 16:0, Monoenoic acids with the double bond in the *trans* conformation (22,62), 18:3n-3, 18:3n-6 (49), 5,11,14-eicosatrienoate, 5,11,14,17-eicosatetraenoate (61), 20:3n-6 (49) and 22:1n-11 (62).

Dietary fatty acids thus appear to be extensively incorporated into the CL class in their intact form, or after one round of "dead end" elongation (*e.g.*, 18:1n-7, 20:2n-6, 20:3n-3), but not after they have been desaturated. For example, 18:2n-6, but not its desaturation/elongation products 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6, is extensively incorporated into CL. Similarly, with essential fatty acid deficiency, 18:1n-9, 18:1n-7 and 16:1n-7 (synthesized endogenously) accumulate to a greater extent than 20:3n-9 and 20:3n-7 (3,15,63).

Physiological consequences of replacing 18:2n-6 with other fatty acids in CL. In the inner mitochondrial membrane, CL is associated with several key enzymes (4–9), the most studied being cytochrome *c* oxidase (10–12). There is evidence from dietary studies (13–15) that modulation of the acyl chain of CL can influence the activity of associated inner mitochondrial membrane proteins. Yamaoka *et al.* (13) found that in rats fed fish oil, 18:2n-6 was largely replaced with 22:6n-3 in hepatic and cardiac CL, and there was a 50% decrease in the activity of cytochrome *c* oxidase. Cytochrome *c* oxidase activity (and possibly other inner mitochondrial membrane enzyme activities) may therefore be modulated by dietary fat intake *via* alterations in the acyl composition of CL. Dietary fat is not known to alter the absolute amount of CL in organs (15,23,31,38,39,64). The results of *in vitro* studies with CL derivatives similarly indicate that the acyl chains of CL, as well as the phosphate moiety and the free 2′-hydroxyl group on the headgroup glycerol, are important in the associations of CL with inner mitochondrial membrane proteins (2,10,11,16–18).

CL, or a related acidic phospholipid may also bind to aPL, possibly bound to plasma β_2-globulin (65). aPL are prevalent in patients with systemic lupus erythematosus and other disorders, and have been correlated to thrombosis, thrombocytopenia, neurological disorders and ischemic heart disease (65). The recent demonstration that passive transfer of aPL to mice can result in fetal loss suggesting that aPL may have direct effects on pregnancy outcome (66). Alteration of the acyl moiety of CL can influence the *in vitro* binding to aPL (19,67,68).

There are several lines of evidence to suggest that the oxidation of CL may affect the activity of associated enzymes: i) Free radical-induced lipid peroxidation of mitochondrial particles is known to lead to the formation of more polar species of CL (as well as PC and PE), and to decreased activities of inner mitochondrial NADH oxidase and NADH-cytochrome *c* reductase (69,70); ii) peroxidation of CL/PC vesicles by addition of superoxide radical ions has been demonstrated to decrease the binding of ferricytochrome *c* to cytochrome *c* oxidase in the tertiary complex (CL-ferricytochrome *c*-cytochrome *c* oxidase) (71); and iii) cytochrome *c*-(Fe^{3+})-H_2O_2 induced peroxidation of mitochondrial membranes may preferentially affect CL, to which cytochrome c binds (72). Malis *et al.* (73) found mitochondrial membranes derived from fish oil fed rats to have enhanced phospholipase A_2-mediated release of fatty acids, and a corresponding reduced NADH coenzyme Q reductase activity after exposure to Ca^{2+} and reactive oxygen species, relative to control membranes. Although the acyl composition of CL was not examined in this study, the feeding of fish oils to rats would result in significant incorporation of 22:6n-3 into mitochondrial CL and to the formation of tetra-docosahexaenoyl molecular species (15). CL rich in 22:6n-3 could thus have been a phospholipid target of the reactive oxygen species in the studies of Malis *et al.* (73).

In summary, we have demonstrated that 18:1n-9 present in olive oil and 22:6n-3 present in fish oil can replace a significant portion of 18:2n-6 in hepatic and cardiac CL of newborn mice. These fatty acids will continue to accumulate during nursing and active feeding of the offspring, provided they are fed in sufficient quantities and for a long enough duration. The major difference between linseed oil (rich in 18:3n-3) and fish oil feeding (rich in 22:6n-3) was that 22:6n-3 accumulated extensively in heart CL only when fed as intact 22:6n-3. Levels of 20:5n-3 and 22:5n-3 were equivalent or greater with linseed oil feeding in CL, PC and PE. These findings may be relevant to human populations who consume diets rich in 18:1n-9 (Mediterraneans), 18:2n-6 (Americans) and 20:5n-3/22:6n-3 (Eskimos and individuals ingesting fish and fish oils). Physiologically, these findings may be important because alterations in CL acyl moiety may influence the activity of associated inner mitochondrial membrane enzymes. The ramifications of altering the acyl composition of CL during development have never been investigated.

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Surface Components of Chylomicrons from Rats Fed Glyceryl or Alkyl Esters of Fatty Acids: Minor Components[1]

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The lipid class, fatty acid and molecular species composition of the minor polar surface components of rat lymph chylomicrons were determined during absorption of menhaden oil and corn oil or of the corresponding fatty acid ethyl esters. In addition to the previously reported minor polar lipids (sphingomyelin, phosphatidylserine, phosphatidylinositol, phosphatidic acid and lysophosphatidylcholine), we identified phosphatidylglycerol, dimethylphosphatidylethanolamine, ceramide and cholesteryl sulfate in the chylomicrons from both oil and ester feeding. The dietary fatty acids were found to be incorporated to a variable extent into the different phospholipid classes, the proportions of which remained the same during both types of feeding. No evidence was obtained for the presence of the minor glycerophospholipids characteristic of the lysosomal membranes (*e.g., bis*-phosphatidic, lyso*bis*phosphatidic and semilyso*bis*-phosphatidic acids), although special efforts were made to identify them. These results indicate that the chylomicrons arising from the monoacylglycerol and phosphatidic acid pathways of triacylglycerol biosynthesis become enveloped in closely similar monolayers of phospholipids. Hence, all triacylglycerols may be secreted from the villus cells *via* a common mechanism as suggested by the previously demonstrated convergence (at the 2-monoacylglycerol stage) of the monoacylglycerol and the phosphatidic acid pathways of mucosal triacylglycerol formation [Yang, Y.L., and Kuksis, A. (1991) *J. Lipid Res. 32*, 1173–1186].
Lipids 27, 613–618 (1992).

We have obtained evidence for an apparent convergence (at the 2-monoacylglycerol stage) of the phosphatidic acid and the monoacylglycerol pathways of triacylglycerol biosynthesis in the rat intestinal mucosa (2). In support of such a common final pathway, we have demonstrated identical apoprotein (3) and major glycerophospholipid (4,5) composition of the chylomicrons generated from oil (monoacylglycerol pathway) and ester (phosphatidic acid pathway) feeding. Since the mechanism proposed for the convergence involves the conversion of the triacylglycerol products of the phosphatidic acid pathway, which are stored and cannot be secreted, into 2-monoacylglycerols by a lysosomal lipase, we have sought evidence of a lysosomal membrane contact by analyzing the minor phospholipids of the chylomicrons for the presence of lysosomal lipids. Lysosomes are known (6) to contain *bis*-monoacylglycerophosphate (*bis*-lysophosphatidic acid) as a characteristic component, which overlaps with phosphatidic acid in the acidic thin-layer chromatography (TLC) systems commonly employed in phospholipid analyses (7). These compounds can be readily resolved in alkaline solvent systems (6). Previous studies (8) had occasionally noted unusually high proportions of phosphatidic acid in rat chylomicrons and prechylomicrons, but a contamination with the polyglycerophosphates had not been considered.

The present study demonstrates that the previously identified "phosphatidic acid" fraction (8) recovered from rat lymph chylomicrons contains significant amounts of other previously unidentified compounds, which can be resolved in an alkaline TLC system, and that the *bis*-phosphatidic acid derivatives are not among them. The results extend the demonstration of the similarity in the surface composition of the chylomicrons from the oil and ester feeding to the minor phospholipids, which had not been assessed previously.

MATERIALS AND METHODS

Animals, surgical procedures and diets. Retired male breeder rats (350–400 g) received 1 mL of menhaden oil or corn oil or the corresponding fatty acid ethyl esters by stomach fistula, and lymph collections were made from the thoracic duct over a period of 2–24 h, as previously described (3,4). Lymph chylomicrons were isolated by ultracentrifugation (3).

Isolation and quantitation of phospholipid classes. Total lipid extracts of chylomicrons were obtained with chloroform/methanol (2:1, vol/vol) and were resolved into neutral and polar lipids by TLC using heptane/isopropyl ether/acetic acid (60:40:4, vol/vol/vol) as the developing solvent (5), which carried the triacylglycerols near the solvent front and retained the phospholipids at the origin. The phospholipids were recovered from the origin by extraction with chloroform/methanol/water/acetic acid (50:39:10:1, by vol) (9). After concentration, the lipids were separated into individual phospholipid classes by TLC using either chloroform/methanol/acetic acid/water (75:45:12:6, by vol) (10) or a double development system (11)—chloroform/methanol/ammonium hydroxide/water (65:35:1:3, by vol) and chloroform/methanol (95:5, by vol) in the same direction. The phospholipid classes from all feedings were identified by reference to standards and those from corn oil feeding also were analyzed by fast atom bombardment-mass spectrometry (FAB-MS). The phospholipid clases were quantitated on the basis of the fatty acid content using heptadecanoic acid as an internal standard. Gel scrapings containing individual phospholipid classes were subjected to acidic methanolysis using 6% H_2SO_4 in methanol at 80°C for 2 h. After addition of the internal standard, the fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) (5). Cholesteryl sulfate was quantitated by measuring cholesterol in relation to the content of dimethylphosphatidylethanolamine (DMPE).

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Abbreviations: CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; FAB-MS, fast atom bombardment-mass spectrometry; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; GP, glycerophosphate; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SPH, sphingomyelin; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

FAB-MS. Fast atom bombardment-mass spectra were obtained using a VG-Analytical ZAB-SE instrument with a VG-11/250 data system (VG Analytical Ltd., Manchester, England). The samples were dissolved in chloroform, and triethylamine was used as matrix for negative ion analysis. The bombardment was effected with xenon atoms at 8 kV anode potential and 1.2 mA anode current.

Acetolysis. In order to determine if the unknown phospholipids contained one or two fatty acids per glycerol molecule, various samples were subjected to acetolysis (12). The products of acetolysis were separated by TLC using petroleum ether/diethyl ether (4:1 by vol) as mobile phase.

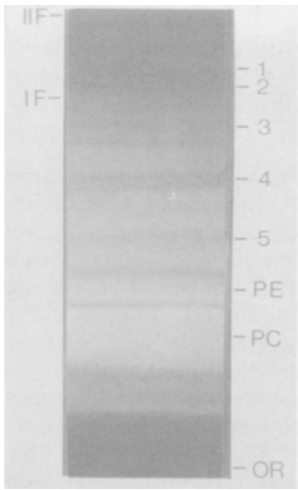

FIG. 1. TLC separation of total phospholipids from lymph chylomicrons. OR, origin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; 1, ceramides; 2, monoacylglycerols plus hydroxyceramides; 3, unknown phospholipid; 4, dimethylphosphatidylethanolamine plus cholesteryl sulfate; 5, phosphatidylglycerol. IF, first solvent front; IIF, second solvent front. Solvent system: First, chloroform/methanol/NH_4OH/water (65:35:1:3, by vol); second, chloroform/methanol (95:5, vol/vol).

RESULTS

Identification of minor polar lipids. Figure 1 indicates the presence of five fast moving bands in the basic TLC system used to examine the minor phospholipids in the chylomicrons from corn oil feeding. These bands migrate close to the solvent front in the acidic TLC system, and in the past apparently have been pooled with phosphatidic acid (8). The R_f values of the unknowns (TLC Bands 3 and 4) correspond closely to those of standard *bis*-monoacylglycerodiacylglycerophosphate (*bis*-semilysophosphatidic acid) and *bis*-monoacylglycerophosphate, respectively. However, acetolysis failed to reveal any monoacylglycerol diacetates, thus eliminating the possible presence of these polyglycerophosphates as major components of these fractions. Only diacylglycerol acetate (TLC Band 3) and diacylglycerol and cholesteryl acetate (TLC Band 4), and diacylglycerol acetate (TLC Band 5) were present as confirmed by gas chromatography/mass spectrometry (GC/MS). The triacetylglycerol also expected in TLC Band 5 was lost due to evaporation. TLC Bands 1 and 2 were rechromatographed in a neutral system, where they could be shown to run with R_f values corresponding to ceramides (TLC Band 1) and monoacylglycerols (TLC Band 2), and a component more polar than monoacylglycerols. The identity of the ceramides and monoacylglycerols was confirmed by GLC (13). Finally, all the minor polar lipid fractions from the initial TLC separation were examined by FAB-MS. TLC Band 1 gave a FAB mass spectrum with prominent $[M-H]^-$ ions at *m/z* 536 and 648, indicating the presence of palmitoyl and lignoceroyl sphingosines, respectively. Other species corresponding to the 22:0, 23:0 and 24:1 amides were present also. These species matched those reported in the literature for the free ceramides of rat intestinal mucosal lipids (14). TLC Band 2, which corresponded in R_f value to *bis*-phosphatidic acid, gave a FAB mass spectrum with prominent $[M-H]^-$ ions at *m/z* 554 and 666, indicating the presence of palmitoyl and lignoceroyl hydroxysphinganine, respectively. Other species corresponding to the 22:1, 22:0, 18:0 and 20:0 hydroxysphinganines also were present. The composition of the hydroxyceramides from rat intestine has been reported (14). The FAB-MS of TLC Band 3, which corresponded in R_f value to the *bis*-semilysophosphatidate, remained unidentified although some characteristics of a glycerophospholipid could be discerned. TLC Band 4, which corresponded in R_f value to *bis*-monophosphatidate (*bis*-lysophosphatidate), gave an FAB mass spectrum with prominent $[M-H]^-$ ions at *m/z* 742 and 770, which indicated the presence of the 16:0–18:2 and 18:0–18:2 species of DMPE, respectively. The fragmentation ions at *m/z* 255, 279, 281 and 283 corresponded to the $[RCOO]^-$ ions of 16:0, 18:2, 18:1 and 18:0 fatty acids. The fragment ions $[PA-H]^-$ at *m/z* 671 and 699 indicated the presence of 16:0–18:2 and 18:0–18:2 diacylglycerophosphates, or phosphatidic acids (PA), respectively. In addition, this TLC band contained a prominent ion $[M-H]^-$ at *m/z* 465, which corresponded to cholesteryl sulfate. The presence of cholesteryl sulfate in this TLC band was confirmed by a GC/MS demonstration of cholesteryl acetate among acetolysis products of this fraction (results not shown). The complex FAB-MS of TLC Band 4 is given in Figure 2. The FAB mass spectrum of TLC Band 5, which migrated between cardiolipin (CL) and phosphatidylethanolamine (PE), gave prominent ions $[M-H]^-$ at *m/z* 745 and 773, which corresponded to the 16:0–18:2 and 18:0–18:2 species of phosphatidylglycerol (PG), respectively. The fragment ions at *m/z* 255, 279, 281 and 283 were due to the $[RCOO]^-$ ions of 16:0, 18:2, 18:1 and 18:0 fatty acids. The fragment ions $[PA-H]^-$ at *m/z* 671 and 699 are due to the 16:0—18:2 and 18:0—18:2 diacylglycerophosphates (GP) or phosphatidic acids, while the fragment ions $[GP-H]^-$ at *m/z* 153 and 171 are due to glycerophosphate. The FAB-MS of PG was similar to the FAB-MS published previously for natural PG (15).

Phospholipid class composition. Table 1 gives the relative proportions of the phospholipid classes isolated from the chylomicrons during the peak of absorption (2–8 h) of menhaden oil and its fatty acid ethyl esters. The values represent best estimates derived from TLC separations obtained with the acidic and basic solvent systems and GLC following appropriate derivatization. In both instances the major components are phosphatidylcholine (PC; 80%) and PE (8%), with much smaller amounts of phosphatidylinositol (PI; 2.5%), phosphatidylserine (PS;

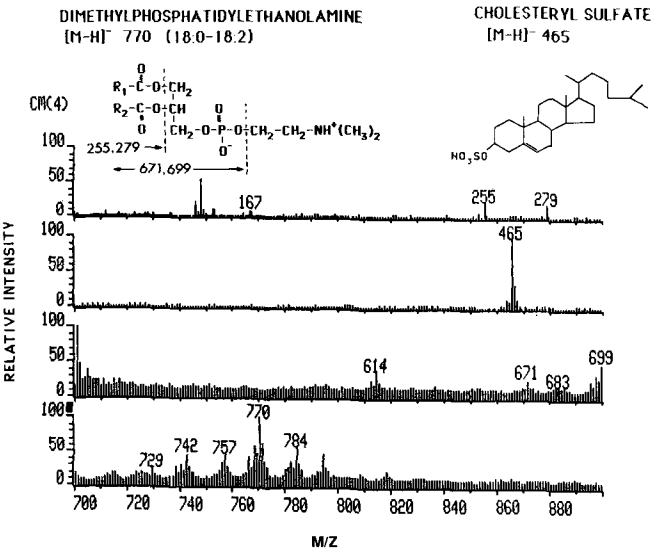

FIG. 2. FAB-MS of DMPE and cholesteryl sulfate. The general structure and cleavage fragments are indicated in the Figure. The ions at *m/z* 742 and 770 correspond to the parent ions of the 16:0-18:2 and 18:0-18:2 species of DMPE, respectively. The ions at *m/z* 671 and 699 correspond to the fragment ions of the corresponding species of PA. The fragment ions at *m/z* 225, 279, 281 and 283 correspond to the 16:0, 18:2, 18:1 and 18:0 fatty acids. The prominent ion at *m/z* 465 is due to cholesteryl sulfate, which was also present in this TLC fraction. FAB-MS conditions are as given under Methods. The individual panels are displayed independently of each other.

TABLE 1

Relative Proportions of Polar Surface Lipid Classes in Chylomicrons from Rats Receiving Menhaden Oil or Corresponding Fatty Acid Ethyl Esters[a]

Polar lipid classes	Source of chylomicrons (mole %)	
	Oil-fed	Ester-fed
Phosphatidylcholine	78.7 ± 1.2	80.6 ± 1.0
Phosphatidylethanolamine	8.1 ± 1.7	7.7 ± 0.2
Phosphatidylinositol	2.7 ± 0.5	2.5 ± 0.4
Phosphatidylserine	0.1 ± 0.0	0.1 ± 0.0
Phosphatidic acid	1.1 ± 0.3	0.9 ± 0.2
Sphingomyelin	0.5 ± 0.2	0.5 ± 0.1
Lysophosphatidylcholine	1.0 ± 0.1	1.4 ± 0.1
Phosphatidylglycerol	1.6 ± 0.2	1.3 ± 0.2
Dimethylphosphatidylethanolamine	1.3 ± 0.5	1.0 ± 0.2
Cholesteryl sulfate	1.5 ± 0.4	1.4 ± 0.5
Ceramides	0.3 ± 0.1	0.3 ± 0.1
Hydroxyceramides[b]	0.2 ± 0.1	0.2 ± 0.0
Monoacylglycerols	0.4 ± 0.3	0.3 ± 0.1
Unknown phospholipid	2.4 ± 1.0	1.9 ± 0.7
PC/FC ratio	8.6 ± 2.0	7.4 ± 1.1

[a]PC/FC, phosphatidylcholine/free cholesterol, X ± SD from 4-6 determinations.
[b]Acylated hydroxysphinganines.

0.1%), PA (1%), sphingomyelin (SPH; 0.5%), lysophosphatidylcholine (LPC; 1%), PG (1.5%) and DMPE (1%). In addition, the chylomicrons contain other polar lipids, such as cholesteryl sulfate (1.5%), ceramide (0.3%), hydroxyceramides (0.2%) and an unidentified minor phospholipid (2%). There are no significant differences between the major phospholipids of chylomicrons when menhaden oil was fed intact or as its fatty acid ethyl ester. Except for the contribution of the unknown and the minor lipids, these values are comparable to those observed earlier during mustard seed oil feeding (4).

Table 2 compares the fatty acid composition of SPH and LPC in the chylomicrons collected at the peak of absorption (2-8 h) of menhaden oil and its fatty acid ethyl ester feeding. Both SPH and LPC have high proportions of 16:0 (50% for SPH and 37% for LPC), 18:0 (17% for SPH and 33% for LPC), and small amounts of 14:0, 16:1, 18:1 and 18:2, along with very small amounts of the polyunsaturated fish oil fatty acids.

Table 3 compares the fatty acid composition of PG and DMPE from chylomicrons collected 2-8 h after menhaden oil or its fatty acid ethyl ester feeding. The oil feeding resulted in more dietary fatty acids in these phospholipids, as indicated by the higher proportion of 18:2 detected and attributed to an incomplete clearance of corn oil used in pre-feeding (5). In other respects, both feedings show comparable effects on the fatty acids of PG and DMPE.

Table 4 gives the fatty acid composition of PA and the unknown lipid fraction. PA was isolated from just above the origin during the phospholipid resolution in a basic TLC system. There are minor differences between the two feedings in the fatty acid composition of these esters. The major acids are of endogenous origin, with very small amounts of the dietary polyunsaturated fatty acids being present. The identity and significance of the unknown lipid requires further investigation.

The ceramides of chylomicrons collected during the peak absorption of menhaden oil and its ethyl ester showed very similar fatty acid composition (data not shown). The two types of ceramides contain significant proportions of 14:0, 16:0 and 18:1, in addition to the saturated long-chain acids. The small amounts of the polyunsaturated dietary fatty acids recovered from these ceramides probably represented minor contamination with glycerophospholipids.

DISCUSSION

The composition of the common chylomicron phospholipids established in this study is comparable to that observed earlier by Myher *et al.* (4) during mustard seed oil absorption. Further, these values are similar to those obtained by Redgrave (8), who reported 6% PA. In the present study, the sum of PA plus minor low polarity phospholipids accounted for 6.4% of total. The phospholipid class composition of the chylomicrons clearly differs from that of the mucosal microsomes reported by Redgrave (8) as 45% PC, 20% PE, 16% LPC, 8% SPH and 8% PI, as well as from that of the prechylomicrons, which showed intermediate composition between that of the microsomes and the chylomicrons, except for 10% PA. The PA levels of the chylomicrons or intestinal mucosal cells analyzed in our laboratory never exceeded 1%. Apparently phosphorus containing lipids other than PA had been included in the higher estimates, as suggested by the present study. The microsomal PE also contained 11-16% alkylacyl and 4-8% alkenylacyl subclasses (16), which occurred in the chylomicrons to less than 5-6% (5). Likewise, the phospholipids of the chylomicrons are different from those of whole villus cells (17) and of rat plasma (18). The

TABLE 2

Fatty Acid Composition of SPH and LPC of Chylomicrons from Rats Receiving Menhaden Oil or Corresponding Fatty Acid Ethyl Esters[a]

Fatty acids	SPH (mole %)		LPC (mole %)	
	Oil-fed	Ester-fed	Oil-fed	Ester-fed
14:0	5.9 ± 2.2	4.2 ± 2.3	2.6 ± 0.9	1.6 ± 0.5
15:0	1.7 ± 0.6	1.3 ± 0.6	1.0 ± 0.3	0.6 ± 0.2
16:0	48.9 ± 6.4	55.1 ± 2.4	37.5 ± 1.7	37.1 ± 3.3
16:1n-7	1.6 ± 0.2	1.2 ± 0.5	2.1 ± 0.4	1.1 ± 0.4
16:2n-4	1.7 ± 1.1	1.2 ± 0.8	0.9 ± 0.4	0.5 ± 0.1
18:0	16.4 ± 1.1	17.1 ± 3.1	32.0 ± 8.3	34.5 ± 3.1
16:3n-4	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
18:1n-9	4.7 ± 1.2	3.8 ± 0.6	5.9 ± 0.4	5.3 ± 0.5
18:1n-7	1.5 ± 0.1	1.2 ± 0.1	2.0 ± 0.4	2.1 ± 0.3
16:4n-1	1.7 ± 0.6	2.1 ± 1.4	1.0 ± 0.5	0.9 ± 0.4
18:2n-6	4.8 ± 1.6	3.6 ± 0.8	6.6 ± 1.5	7.4 ± 0.9
18:3n-3	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.5	0.2 ± 0.1
20:1n-9	0.3 ± 0.1	0.8 ± 0.9	0.4 ± 0.1	0.5 ± 0.1
18:4n-3	1.8 ± 1.1	1.7 ± 0.4	0.6 ± 0.3	0.5 ± 0.1
20:3n-6	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	0.4 ± 0.2
20:4n-6	2.5 ± 1.4	2.1 ± 0.3	3.5 ± 0.2	3.9 ± 0.6
20:4n-3	0.0 ± 0.0	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
20:5n-3	2.2 ± 0.9	1.8 ± 0.9	2.9 ± 0.4	1.5 ± 0.3
22:5n-3	0.4 ± 0.3	0.6 ± 0.3	0.4 ± 0.1	0.4 ± 0.1
22:6n-3	0.9 ± 0.6	1.4 ± 0.4	1.6 ± 0.8	1.5 ± 0.2
Other	2.5	1.5		

[a]X ± SD from six determinations.

TABLE 3

Fatty Acid Composition of the PG and DMPE of Chylomicrons from Rats Receiving Menhaden Oil or Corresponding Fatty Acid Ethyl Esters[a]

Fatty acids	PG (mole %)		DMPE (mole %)	
	Oil-fed	Ester-fed	Oil-fed	Estere-fed
14:0	2.9 ± 0.7	3.9 ± 1.3	3.5 ± 1.6	3.4 ± 1.3
15:0	0.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.3	0.5 ± 0.3
16:0	18.5 ± 2.5	22.7 ± 0.9	16.0 ± 4.7	16.8 ± 2.6
16:1n-7	2.2 ± 0.8	5.0 ± 1.0	2.5 ± 0.7	4.3 ± 2.0
16:2n-4	0.5 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	0.8 ± 0.2
18:0	23.6 ± 2.4	23.7 ± 1.5	23.6 ± 2.3	23.8 ± 1.6
16:3n-4	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
18:1n-9	10.9 ± 0.8	9.1 ± 0.6	9.6 ± 1.4	9.7 ± 1.9
18:1n-7	3.8 ± 0.7	4.8 ± 0.8	2.7 ± 0.6	3.3 ± 0.5
16:4n-1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.5 ± 0.1
18:2n-6	15.2 ± 1.7	8.7 ± 0.8[b]	16.5 ± 4.8	10.2 ± 1.8[b]
18:3n-3	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.1
20:1n-9	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
18:4n-3	0.6 ± 0.2	0.6 ± 0.1	0.2 ± 0.3	0.7 ± 0.2
20:3n-6	0.6 ± 0.1	0.4 ± 0.0	0.6 ± 0.3	0.6 ± 0.1
20:4n-6	6.7 ± 1.1	5.0 ± 0.1	8.1 ± 1.5	7.2 ± 0.9
20:4n-3	0.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.4	0.5 ± 0.2
20:5n-3	3.0 ± 1.2	5.6 ± 1.1	4.6 ± 1.1	7.3 ± 2.5
22:5n-3	1.2 ± 0.2	0.9 ± 0.2	1.0 ± 0.3	1.1 ± 0.2
22:6n-3	7.9 ± 2.2	6.1 ± 0.8	7.8 ± 2.4	7.8 ± 0.9

[a]X ± SD from 4-5 determinations.
[b]$P < 0.05$ as compared to oil feeding.

present results clearly exclude the origin of the major chylomicron phospholipids by transfer from any of the above sources as well as by extensive equilibration with any plasma lipoproteins that may have filtered into lymph. These findings would make a significant lysosomal contribution unlikely in view of the absence of the polyglycerophospholipids from the chylomicrons.

In view of the above results, it is necessary to look to other explanations for the origin of the peculiar composition of chylomicron phospholipids. The relative absence of PE and the acidic phospholipids from the surface of the chylomicrons would suggest a selective loss based on monolayer stability during the passage of the chylomicrons from the site of initial assembly to the point of secretion. This would result in a preferential retention of PC, which is known to more readily form phospholipid

TABLE 4

Fatty Acid Composition of PA and Unknown Phospholipid of Chylomicrons from Rats Receiving Menhaden Oil or Corresponding Fatty Acid Ethyl Esters[a]

Fatty acids	PA (mole %)		Unknown PL (mole %)	
	Oil-fed	Ester-fed	Oil-fed	Ester-fed
14:0	3.3 ± 0.8	2.5 ± 0.5	3.2 ± 1.8	3.7 ± 1.1
15:0	1.1 ± 0.4	0.7 ± 0.1	0.3 ± 0.1	0.6 ± 1.1
16:0	17.6 ± 3.4	17.7 ± 2.5	14.7 ± 3.1	17.2 ± 2.8
16:1n-7	2.4 ± 0.9	2.0 ± 1.0	2.8 ± 0.7	4.6 ± 1.7
16:2n-4	1.4 ± 0.2	1.1 ± 0.2	0.5 ± 0.1	0.9 ± 0.1
18:0	23.9 ± 1.0	27.1 ± 2.4	22.4 ± 2.6	22.7 ± 2.3
16:3n-4	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.5 ± 0.1
18:1n-9	7.0 ± 1.0	6.5 ± 2.2	10.2 ± 0.4	9.8 ± 3.0
18:1n-7	2.4 ± 0.7	2.8 ± 0.8	3.0 ± 0.7	3.4 ± 0.5
16:4n-1	1.1 ± 0.2	0.9 ± 0.3	0.3 ± 0.1	0.5 ± 0.1
18:2n-6	9.2 ± 3.3	8.3 ± 1.3	15.8 ± 5.1	9.5 ± 2.0
18:3n-3	0.3 ± 0.0	1.0 ± 0.8	0.4 ± 0.1	0.4 ± 0.1
20:1n-9	0.4 ± 0.2	0.5 ± 0.1	0.7 ± 0.2	0.7 ± 0.1
18:4n-3	0.9 ± 0.1	0.8 ± 0.4	0.6 ± 0.1	0.7 ± 0.3
20:3n-6	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.5 ± 0.1
20:4n-6	9.9 ± 1.9	9.6 ± 1.4	8.4 ± 1.7	7.0 ± 0.9
20:4n-3	0.3 ± 0.0	0.5 ± 0.3	0.7 ± 0.2	0.7 ± 0.1
20:5n-3	5.9 ± 1.6	5.1 ± 1.7	4.8 ± 1.7	7.8 ± 3.0
22:5n-3	1.8 ± 0.5	1.9 ± 0.6	1.2 ± 0.4	0.8 ± 0.4
22:6n-3	10.6 ± 4.2	10.6 ± 2.2	8.7 ± 2.8	7.9 ± 1.4

[a]X ± SD from 4-6 determinations.

monolayers at the oil-water interface (19) than PE, PS, PI or LPC (20). It is not known whether this effect extends to the oil-water interface involved in the chylomicron secretion and to what extent the monolayer stability may be influenced by interaction with apoprotein B and other apoproteins also present on the chylomicron surface.

In an earlier study O'Doherty *et al.* (21) demonstrated the need of PC during fat absorption in choline deficient rats with bile fistula, and suggested a requirement for a continued supply of a phospholipid surfactant. More recently, Vance *et al.* (22) reported evidence for a possible need of PC biosynthesis during VLDL secretion by rat liver. The present results obtained on the minor polar lipids of the chylomicrons are consistent with a biosynthesis of surface active components, because several of the newly identified lipids are precursors of phospholipids and sphingolipids. Thus, DMPE is an intermediate in PC synthesis *via* PE methylation pathway, which is known to be active in endoplasmic reticulum and the Golgi of hepatocytes during very low density lipoprotein (VLDL) secretion (22,23). It is believed to contribute some 20–40% of the cellular PC. The methylation pathway of PE has been claimed to be activated in the liver of the choline deficient rat (24). The fatty acid composition of DMPE was similar to that of PE collected at the same time and reported previously (5). This was in agreement with the work of Vance (23), which had shown that the DMPE of the rat liver VLDL possesses a fatty acid composition similar to that of PE. The fatty acid composition of PC is not achieved until after complete methylation of PE to PC and a probable acyl exchange. A biosynthetic involvement of DMPE is favored by the rapid appearance of dietary fatty acids in this lipid during fat absorption, as shown in the present work.

The presence of free ceramides in the chylomicrons would suggest a possible role in the biosynthesis of glycosphingolipids, which are known to be synthesized in mucosal cells (25), although they were not analyzed in the present work. These ceramides are less likely to be precursors of sphingomyelin, the chylomicron content of which is not increased. The isolation of substantial amounts of cholesteryl sulfate from the chylomicrons is a new finding, but fully consistent with the hypothetical need for surfactants. Cholesteryl sulfate has been reported in plasma low density lipoproteins by Epstein *et al.* (26) and Nakamura *et al.* (27). It is known to serve as a membrane stabilizer (27). In recessive X-linked ichtyosis patients, which lack cholesterol sulfatase, the abnormal amount of the accumulating steroid causes desquamation, as characterized by scaly skin and corneal opacity (27). Hence, the specific biosynthesis of PC, reflecting a need for surfactant during chylomicron formation and secretion would appear to provide the most logical explanation for its enrichment in the chylomicrons, along with other minor surfactants. We have previously demonstrated a general increase in glycerophospholipid synthesis in response to fat absorption by villus cells (17). The present study indicates that a further investigation of surfactant synthesis during chylomicron formation and secretion would be justified.

The present study provides further evidence for the similarity in the surface lipids of chylomicrons formed *via* the phosphatidic acid and the monoacylglycerol pathways of triacylglycerol biosynthesis. These results lend credibility to the hypothesis that the two synthetic pathways yield only one final triacylglycerol product which is packaged and secreted as chylomicrons (2).

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Effects of Dietary Saturated Fat on Erucic Acid Induced Myocardial Lipidosis in Rats

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Male Sprague-Dawley rats were fed for one week diets containing 20% by weight fat/oil mixtures with different levels of erucic acid (22:1n-9) (~2.5 or 9%) and total saturated fatty acids (~8 or 35%). Corn oil and high erucic acid rapeseed (HEAR) oil were fed as controls. The same hearts were evaluated histologically using oil red O staining and chemically for cardiac triacylglycerol (TAG) and 22:1n-9 content in cardiac TAG to compare the three methods for assessing lipid accumulation in rat hearts. Rats fed corn oil showed trace myocardial lipidosis by staining, and a cardiac TAG content of 3.6 mg/g wet weight in the absence of dietary 22:1n-9. An increase in dietary 22:1n-9 resulted in significantly increased myocardial lipidosis as assessed histologically and by an accumulation of 22:1n-9 in heart lipids; there was no increase in cardiac TAG except when HEAR oil was fed. An increase in saturated fatty acids showed no changes in myocardial lipid content assessed histologically, the content of cardiac TAG or the 22:1n-9 content of TAG at either 2.5 or 9% dietary 22:1n-9. The histological staining method was more significantly correlated to 22:1n-9 in cardiac TAG ($r = 0.49$; $P < 0.001$) than to total cardiac TAG ($r = 0.40$; $P < 0.05$). The 22:1n-9 content was highest in cardiac TAG and free fatty acids. Among the cardiac phospholipids, the highest incorporation was observed into phosphatidylserine, followed by sphingomyelin. With the addition of saturated fat, the fatty acid composition showed decreased accumulation of 22:1n-9 and increased levels of arachidonic and docosahexaenoic acids in most cardiac phospholipids, despite decreased dietary concentrations of their precursor fatty acids, linoleic and linolenic acids.
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Erucic acid (22:1n-9) in the diet has been shown to cause myocardial lipidosis, particularly in rats, and to a lesser extent in other species (1–4). On continuous feeding, lipidosis reaches a maximum at 4 to 7 days and decreases thereafter. Diets with >10% 22:1n-9 in the oil/fat portion result in increased myocardial lipidosis as assessed histologically with appropriate fat-soluble dyes, increased triacylglycerol (TAG), and incorporation of 22:1n-9 into cardiac lipid classes.

The occurrence of myocardial lipidosis is less consistent when diets containing <10% 22:1n-9 in the oil portion are fed, and results are dependent on which method is used to assess the lipid accumulation. Some investigations have reported increased myocardial lipidosis with 10% 22:1n-9 in the dietary oil (5,6); others with 8% (7), 6% (8), 5% (9), and 2% (10–12). Furthermore, some researchers have reported myocardial lipidosis in rats fed high fat diets containing no 22:1n-9 with such oils as corn (11–13), peanut (8,9), soybean (14) and sunflower (7,15), and with lard (16) and lard/corn oil (12).

Dietary saturated fatty acids (SFA) have been shown to improve growth rate in rats (17–19), reduce incidence and/or severity of myocardial necrosis in male rats (19–22), improve pathogenic characteristics in ducklings (18), as well as reduce myocardial lipidosis in rats fed diets rich in 20:1n-9, but not with 22:1n-9 (23). The reason may have been that the dietary content of 22:1n-9 was too high in these diets, *i.e.*, 30 and 70% (23). No other studies were designed to test the effects of different levels of SFA on myocardial lipidosis in rats in the presence of much lower dietary concentrations of 22:1n-9, and the effects of TAG and the composition of fatty acids in the heart.

The present study was designed to test if SFA would influence the incidence and/or severity of myocardial lipidosis in male rats fed <10% 22:1n-9, to determine if dietary SFA influence the relative concentration of other fatty acids in cardiac lipids in the presence of different levels of dietary 22:1n-9, and to compare the results of histological and chemical assays.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats (Charles River Canada Inc., St. Constant, Quebec, Canada), weighing 124.1 ± 0.7 g, were fed one of six diets (10 rats/diet) for one week. The test oil mixtures were prepared by mixing canola oil, high erucic acid rapeseed (HEAR) oil and cocoa butter in proportions to give oils with about 2.5 and 9% 22:1n-9, each with a low (8%) and a high content (35%) of total SFA. The ratios of oils used and the fatty acid composition of all test oils are shown in Table 1. Semisynthetic diets were prepared containing oil at 20% by weight as described previously (19); diets were fed *ad libitum*.

After one week on the test diet, rats were anesthetized with CO_2 and killed by decapitation. Hearts were removed immediately. Hearts were bisected longitudinally along the median plane. Half the heart was fixed in 10% neutral buffered formalin and the other half placed on dry ice for total lipid extraction (24). Following formalin fixation, the tissue was frozen in liquid nitrogen, sectioned longitudinally on a cryostat at −20°C and stained with oil red O. The percent of tissue area stained was estimated by examining fields under 200-fold magnification, judging the percent of each field involved in lipidosis and averaging the percentages, to arrive at a percent involvement for each heart section.

The cardiac lipids were separated by 3-directional thin-layer chromatography (TLC) (25) on silica gel H plates (Analtech Inc., Newark, DE), first direction, chloroform/methanol/28% aqueous ammonia (65:25:5, vol/vol/vol); second direction, chloroform/methanol/acetone/acetic acid/water (50:10:20:15:5, by vol); and third direction (for

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Abbreviations: ANOVA, analysis of variance; DPG, diphosphatidylglycerol; FFA, free fatty acids; GLC, gas-liquid chromatography; HEAR, high erucic acid rapeseed; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SP, sphingomyelin; TLC, thin-layer chromatography; TAG, triacylglycerol; UV, ultraviolet.

TABLE 1

Fatty Acid Composition of Dietary Oils

Fatty acids	Oil mixtures[a]				HEAR oil	Corn oil
	A	B	C	D		
16:0	4.6	13.9	4.6	14.5	3.2	12.3
18:0	2.1	18.9	2.1	18.4	1.2	2.1
20:0	0.9	1.0	0.9	1.0	0.8	0.5
22:0	0.5	0.4	0.6	0.4	0.7	0.1
24:0	0.2	0.2	0.3	0.2	0.3	0.1
Σ Saturated	8.4	34.7	8.5	34.6	6.3	15.2
16:1n-7	0.2	0.2	0.2	0.2	0.2	0.2
18:1n-9	56.5	45.6	51.3	39.3	19.0	26.1
18:1n-7	3.2	1.9	2.8	1.5	0.8	0.6
20:1n-9	2.2	1.3	2.7	1.7	6.3	0.3
22:1n-9	2.9	2.4	10.1	8.7	42.9	0
24:1n-9	0.4	0.2	0.5	0.3	1.1	0
Σ Monoenoic	65.3	51.7	67.7	51.7	70.3	27.2
18:2n-6	18.2	10.2	16.5	9.5	14.3	56.7
18:3n-3	7.0	3.1	6.1	3.2	8.4	0.6

[a]Diets contained two different levels of erucic acid (22:1n-9) and two different levels of saturated fatty acids (SFA). The dietary oil mixtures were prepared by mixing canola oil (0.8% 22:1n-9), high erucic acid rapeseed (HEAR) oil (42.9% 22:1n-9), and cocoa butter in the ratio of 96:4:0 (A), 54:4:42 (B), 81:19:0 (C) and 39:19:42 (D) by weight, respectively.

neutral lipids only) hexane/diethyl ether/acetic acid (85:15:1, vol/vol/vol). The lipid classes were visualized under ultraviolet (UV) light after spraying the plates with a 2′,7′-dichlorofluorescein solution in methanol. Lipid fractions were scraped off the plate, and methyl heptadecanoate (NuChek Prep, Elysian, MN) was added as internal standard (26). The lipid/silica gel mixtures were transmethylated with anhydrous HCl/CH_3OH (5% by wt). The resultant fatty acid methyl esters were purified by TLC (hexane/diethyl ether/acetic acid, 85:15:1, vol/vol/vol) and quantitated by gas liquid chromatography (GLC) using fused silica capillary columns (27). Cholesterol was determined colorimetrically (26).

Analysis of variance was used to assess treatment differences; factors in the model were level of dietary SFA, 22:1n-9 and their interaction. Treatment effects were considered significant at $P < 0.05$. Because of heterogeneity of variances, data were also analyzed after a logarithm transformation. Since the statistical inferences were unchanged, only the results based on the original data were presented. The relationships among several of the response criteria were evaluated by computing the partial correlation coefficient after adjusting for treatment effects.

RESULTS

There were no significant differences in weight gain of rats fed the four oil mixtures and corn oil (55.3 ± 2.2 g). Rats fed the HEAR oil diet gained significantly ($P < 0.05$) less (49.4 ± 1.8 g). The heart weights were similar for all groups (0.72 ± 0.2 g). Total heart lipids were higher for the HEAR oil group (59.5 ± 3.2 mg/g wet weight) compared to all other groups, which showed no significant differences among each other (27.7 ± 1.0 mg/g wet weight). Among the rats fed the 4 test oil mixtures, there were no significant effects on weight gain, heart weight or total heart lipids, related to their dietary differences in 22:1n-9 and total SFA.

The histological results indicated trace myocardial lipidosis in most rats fed corn oil (Table 2). Compared to rats fed corn oil, the incidence and severity was slightly higher in rats fed oils with about 2.5% 22:1n-9, but the differences were not significant ($P > 0.05$). Oils with about 9% 22:1n-9 produced significantly increased myocardial lipidosis compared to rats fed oils with about 2.5% 22:1n-9. The areas most affected were the right and left ventricles near the base of the heart. Among the four test oil mixtures, there was a significant effect ($P < 0.001$) for dietary 22:1n-9, but not for total SFA. In contrast, myocardial lipidosis in HEAR oil-fed rats was very extensive throughout the whole heart. For an illustration, see Figure 2 in reference (28).

The cardiac lipid classes were not significantly different among the diets (results not shown), except for TAG which was significantly increased in HEAR oil-fed rats (Table 2). Among the four test oil mixtures, there was no significant effect related to dietary 22:1n-9 or total SFA, but there was a significant interaction ($P < 0.05$).

The 22:1n-9 content in cardiac TAG was directly related to the dietary content of 22:1n-9 (55 to 89% of diet), but the content of SFA had no effect (Table 2). This was not the case among the cardiac phospholipids, which showed a significant decrease in 22:1n-9 accumulation in several of the major cardiac phospholipids with an increase in dietary SFA (Table 3). The exception was cardiac sphingomyelin (SP), which showed a significant increase of 22:1n-9 with increased SFA.

Dietary 22:1n-9 accumulated in all lipid classes (Table 3). The highest accumulation was found in cardiac TAG, free fatty acids (FFA), phosphatidylserine (PS) and SP. Generally, the increase in dietary 22:1n-9 did not affect the relative concentration of other cardiac fatty acids including total SFA and arachidonic acid (20:4n-6), but the C_{22} polyunsaturated fatty acids (PUFA) content appeared to decrease, on occasion significantly. The addition of SFA to the oil mixtures, however, showed generally

TABLE 2

Comparison of Histological Staining (using oil red O), Triacylglycerol (TAG) and Erucic Acid (22:1n-9) Content in TAG of Hearts of Rats Fed for One Week the Test Oils at 20% by Weight of the Diet

Dietary oils[a]	Content in diet		Histological stain[b]		TAG[c]	22:1n-9 in TAG[c]
	22:1n-9	SFA	Incidence	Severity		
	(%)	(%)		(area%)	(mg/g wet wt)	(wt%)
A	2.9	8.4	10/10	$13 \pm 4.8^{1,d}$	$3.7 \pm 0.4^{1,e}$	$1.6 \pm 0.1^{2,e}$
B	2.4	34.7	8/10	14 ± 5.6^{1}	5.0 ± 0.7^{1}	1.4 ± 0.1^{2}
C	10.1	8.5	10/10	44 ± 9.3^{2}	5.8 ± 0.7^{1}	6.8 ± 0.8^{3}
D	8.7	34.6	10/10	56 ± 9.0^{2}	4.4 ± 0.5^{1}	7.6 ± 0.8^{3}
HEAR oil	42.9	6.3	10/10	100^{3}	33.4 ± 3.8^{2}	38.3 ± 1.1^{4}
Corn oil	0	15.2	6/10	3 ± 1.2^{1}	3.6 ± 0.3^{1}	0.1 ± 0.02^{1}
			Significance[g]			
ANOVA of first 4 diets[f]						
Effect of 22:1n-9			n.s.	***	n.s.	***
Effect of SFA			n.s.	n.s.	n.s.	n.s.
Interaction, 22:1n-9 x SFA			n.s.	n.s.	*	n.s.
Partial Person's correlation coefficients adjusted for diets (first 4 diets)[f]:						
Oil red O *vs.* TAG			0.339*			
Oil red O *vs.* 22:1n-9 in TAG			0.488**			
TAG *vs.* 22:1n-9 in TAG			0.118 (n.s.)			

[a]Diets A, B, C and D are mixtures of canola oil (0.8% 22:1n-9), high erucic acid rapeseed (HEAR) oil (42.9% 22:1n-9) and cocoa butter; ratios shown in Table 1. The erucic acid (22:1n-9) and saturated fatty acid (SFA) content of all diets are shown.

[b]Oil red O was the histological lipid stain used. Incidence, number affected/number examined. Severity expressed as average area % of heart tissue affected ± SEM.

[c]Means ± SEM.

[d]Means within a column with different superscript numbers are significantly different at $P < 0.05$.

[e]Number of rat hearts examined was nine for these means only.

[f]Analyses of variance (ANOVA) with exclusion of HEAR oil and corn oil diets.

[g]d.f., Degrees of freedom; n.s., not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

little effect on the relative concentration of other fatty acids, except for the decrease in 22:1n-9 already pointed out, and the expected increase in total SFA of cardiac TAG (Table 3).

Based on the partial correlations, histological staining was highly correlated ($P < 0.01$) with 22:1n-9 in cardiac TAG (Table 2), as well as all other cardiac phospholipids ($P < 0.001$), except SP (results not shown). Furthermore, after adjusting for diets, the histological staining was positively correlated to other monounsaturated fatty acids (18:1n-9 and 20:1n-9) and linoleic acid (18:2n-6) and negatively correlated to SFA (14:0, 16:0, 18:0, 20:0 and 24:0); correlations were often significant for 16:0, 18:2n-6 and 20:1n-9 (results not shown). In cardiac SP, only 18:1n-9 showed a significant ($P < 0.05$) and positive correlation to histological staining.

DISCUSSION

The results of this study indicate that sparsely dispersed lipid droplets were readily detected by histological staining in most rat hearts examined (Table 2). Dietary 22:1n-9 exacerbated lipid droplet formation, but it did not appear to be the only cause, since the hearts of rats fed corn oil, which contained no 22:1n-9, also showed evidence of increased myocardial lipidosis. This confirms earlier observations by some investigators of trace myocardial lipidosis in rats fed high fat diets containing such oils as corn, peanut, soybean and sunflower, all of which contain no 22:1n-9 (7–9,11–15). The histological technique (staining with oil red O) is sensitive and will detect localized lipid accumulation, which in these hearts occurs mainly in the right and left ventricles near the base of the heart. At moderate to low severity of myocardial lipidosis, there are large differences in lipidosis responses between animals on the same diet (see SEM, Table 2), which depends on where the section was taken.

Even though these lipid droplets consist of TAG (1–4), myocardial lipidosis could not be confirmed by chemical analyses as increased TAG, even when the other half of the same heart was investigated. In order to detect increased TAG by chemical analyses, it appears that lipidosis needs to be dispersed over the entire heart, not simply in localized areas as shown by oil red O. Similar results were obtained recently by correlating myocardial lipidosis to cardiac TAG in newborn piglets fed milk replacer diets containing different oils (29). It should be noted that normal hearts contain about 4 mg/g wet weight intracellular TAG present as droplets, which are generally not visible after staining with oil red O and examining the sections using a light microscope. However, the lipid droplets in normal heart tissue can be seen using electron microscopy (9,15,30,31). Based on these results, it would appear that 22:1n-9 increased the droplet size, such that the droplets can be detected with oil red O, without significantly increasing the level of cardiac TAG (Table 2).

TABLE 3

Changes in the Relative Composition of Selected Fatty Acids in Heart Tissue Lipid Classes

Fatty acid	Oil Mixtures[a]				LSD[b]	Significance[c]	
Lipid class[d]	A	B	C	D	($P < 0.01$)	22:1n-9	SFA
	(n = 9)[e]	(n = 10)	(n = 10)	(n = 10)			
Σ Saturated							
TAG	24.4	32.2	22.5	30.1	5.3	n.s.	***
FFA	47.8	55.9	46.8	51.8	18.8	n.s.	n.s.
PC	47.2	49.3	44.8	47.8	4.6	*	**
PE	33.6	35.8	34.4	35.0	7.0	n.s.	n.s.
DPG	3.9	2.9	2.6	3.0	2.0	n.s.	n.s.
PS	59.6	60.5	58.6	58.1	8.5	n.s.	n.s.
PI	51.9	54.8	52.3	55.1	8.3	n.s.	n.s.
SP	79.4	80.8	78.6	76.5	11.2	n.s.	n.s.
22:1n-9							
TAG	1.6	1.4	6.8	7.6	3.0	***	n.s.
FFA	1.4	1.5	6.1	6.2	3.9	***	n.s.
PC	0.3	0.2	0.8	0.6	0.2	***	***
PE	0.3	0.3	1.0	0.7	0.3	***	*
DPG	0.1	0.1	0.3	0.3	0.1	***	n.s.
PS	1.7	1.4	4.0	3.5	1.0	***	$P = 0.06$
PI	0.1	0.1	0.3	0.3	0.2	***	n.s.
SP	0.5	0.7	1.5	2.2	0.9	***	*
20:4n-6							
TAG	0.9	1.0	0.7	1.0	0.5	n.s.	n.s.
FFA	3.0	3.4	2.9	2.8	2.2	n.s.	n.s.
PC	17.5	20.7	18.2	19.5	1.6	n.s.	***
PE	18.5	19.3	18.0	18.4	2.9	n.s.	n.s.
DPG	2.1	1.5	1.4	1.5	0.9	*	n.s.
PS	5.0	6.0	4.9	6.0	1.9	n.s.	**
PI	22.7	23.5	21.4	23.3	4.2	n.s.	n.s.
Σ C_{22} PUFA							
TAG	1.1	1.0	0.8	1.1	0.7	n.s.	n.s.
FFA	1.9	2.2	1.4	1.8	1.1	*	n.s.
PC	4.8	5.5	4.2	4.6	1.9	$P = 0.05$	n.s.
PE	20.7	22.3	17.8	19.7	7.0	$P = 0.06$	n.s.
DPG	1.7	1.6	1.3	1.5	0.8	n.s.	n.s.
PS	14.3	16.4	13.1	16.1	4.0	n.s.	**
PI	3.3	3.4	2.8	2.7	1.5	*	n.s.

[a]Diets A, B, C and D are mixtures of canola oil (0.8% 22:1n-9), high erucic acid rapeseed (HEAR) oil (42.9% 22:1n-9) and cocoa butter; ratios shown in Table 1. The erucic acid (22:1n-9) and saturated fatty acid (SFA) content of the diets are shown.

[b]LSD, least significant difference. Means within a row which differ by more than the LSD are significantly different at $P < 0.01$.

[c]Significance due to differences in erucic acid (22:1n-9) or saturated fatty acids (SFA). n.s., Not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

[d]For abbreviations of lipid classes see Footnote to title page.

[e]Number of rats/diet.

Erucic acid in cardiac TAG showed a higher correlation with oil red O staining than cardiac TAG (Table 2). However, the value of 22:1n-9 in cardiac TAG should be considered an overestimation of lipid droplets, since 22:1n-9 was incorporated into all cardiac TAG, but not all cardiac TAG was detected histologically using a light microscope. The correlation of oil red O to 22:1n-9 in cardiac TAG would therefore appear to be a reliable indicator, since 22:1n-9 at higher dietary concentrations will cause myocardial lipidosis.

It is evident from the results of this study that dietary SFA do not affect the incidence and/or severity of myocardial lipidosis at either 2.5 or 9% 22:1n-9, as measured by any of the three methods (Table 2). This suggests that 22:1n-9 acts independently in causing myocardial lipidosis at low (<10%; this study) or high (>30%; see ref. 23) concentrations of 22:1n-9. However, even though no discernible effects were observed in myocardial lipidosis and/or accumulation of cardiac TAG, the fatty acid profile of heart lipids showed distinct changes due to increased SFA. These include a general reduction in the incorporation of 22:1n-9 into several cardiac phospholipids, and increased concentrations of 20:4n-6 and C_{22} PUFA (Table 3), despite lower dietary levels of the precursor fatty acids, linoleic (18:2n-6) and linolenic (18:3n-3) acids (Table 1).

The question remains, what is the minimum level of dietary 22:1n-9 that will cause myocardial lipidosis? Such a question might imply that only 22:1n-9 induces myocardial lipidosis; this is not the case. A number of conditions are known to cause accumulation of lipid droplets such as stress, hypoxia, intoxication (9,15), including the first

consumption of maternal milk (13,29) and fasting (32). The problem is further complicated by the fact that vegetable oils fed at 20% by weight of the diet, even though they do not contain 22:1n-9, also cause trace myocardial lipidosis in rats (7-9,11-15) and pigs (29). From several studies using neonatal pigs, it appears that levels of more than 5% 22:1n-9 in the dietary oil caused increased myocardial lipidosis (29). On the other hand, results from this and other studies (9-12) with rats would suggest that levels of about 2 to 3% 22:1n-9 already show increased myocardial lipidosis. Species differences may account for the differences in sensitivity.

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Increments of Dietary Linoleate Raise Liver Arachidonate, But Markedly Reduce Heart n-6 and n-3 Fatty Acids in the Rat

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Four diets containing 20% of energy (en%) as fat and with linoleic acid contents of 1.9, 3.1, 7.7 and 10.1 en%, respectively, were fed to one-month-old male rats for three months. The fatty acid profiles and the levels of the major n-6 and n-3 fatty acids in the lipids of plasma, liver, heart and kidney were measured. We found that with increasing concentrations of 18:2n-6 in the diet, linoleic acid rose in plasma and in all organs, but long-chain n-6 and n-3 fatty acids responded differently. In liver, arachidonic acid increased and n-3 fatty acids were not significantly affected; in heart, both arachidonic and docosahexaenoic acids were progressively reduced; and in kidney, there was no change of n-6 and n-3. The results indicate that incremental changes in dietary linoleate affect the levels of polyunsaturated fatty acids in liver and extrahepatic organs differently.
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The effect of diets with different linoleic acid (LA) contents, on the levels of polyunsaturated fatty acids (PUFA) in tissue lipids has been examined in experimental animals (1,2). Most studies, however, have been aimed mainly at investigating the changes of fatty acid (FA) profiles of tissue and plasma lipids after feeding different types of fats containing markedly different proportions of saturated FA, monounsaturated FA and PUFA (n-6 and n-3). Thus, the relationships between changes in LA, the major essential FA in the diet, and the accumulation of LA and its derivatives in tissues were not specifically investigated. An additional difficulty in evaluating the data available is due to the lower fat content in rodent diets when compared to human diets.

The relationships between the intakes of LA, the most abundant PUFA in the diet and in tissues, and the levels of arachidonic acid (AA), the major product of LA metabolism, in tissues and organs, are of special nutritional interest. Over the last decades, several expert panels have, in fact, recommended an increase in LA intakes up to 7 to 10 percent energy (en%), quite above the minimum requirement. *In vitro* studies indicate a high rate of production of the long-chain, highly unsaturated fatty acids in liver (3–10). However, in feeding studies, it is difficult to predict the relationships between different intakes of LA and the levels of n-6 highly unsaturated fatty acids (HUFA) in tissues and organs, since these appear to change differently in the liver and in other organs.

To explore the relationships between levels of LA in the diet and those of n-6 and n-3 PUFA in different tissues, we have fed defined diets, with a fat content of 10% (w/w), corresponding to 20.6 en%, and LA contents ranging between 2 to 10 en%, to groups of rats. The levels of LA and of the long-chain PUFA of the n-6 and n-3 series in plasma, liver and extrahepatic organs (heart and kidney) have then been measured. The major findings were that increasing levels of dietary LA, within the range of recommended intakes for humans, resulted in elevation of LA in all tissues. AA accumulated in the liver, whereas it decreased in the heart, with LA levels greater than 3.1 en% while docosahexaenoic (DHA) acid decreased progressively with increasing dietary LA levels. In the kidney, PUFA were minimally affected by changes of dietary LA.

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; CE, cholesteryl esters; DHA, docosahexaenoic acid; EFA, essential fatty acids; en%, percent energy; EPA, eicosapentaenoic acid; FA, fatty acids; HUFA, highly unsaturated fatty acids; LA, linoleic acid; PL, phospholipids; PUFA, polyunsaturated fatty acids; TG, triglycerides; GOT, glutamic-oxalacetic transaminase; LDH, lactic dehydrogenase; GPT, glutamic-pyruvic transaminase.

MATERIALS AND METHODS

Animals and diets. Four groups (six animals/group) of male rats of the Charles River strain were fed *ad libitum* the diets under study for a period of three months, starting at one month of age. Animals were individually caged in the first three weeks of treatment, in order to evaluate individual food consumption and body growth; subsequently, they were kept three animals/cage.

The four semisynthetic diets used were prepared according to AIN (AIN-76™) recommendations (Piccioni S.p.A., Gessate, Italy), had adequate protein, carbohydrate, vitamin and micronutrient contents, and contained 10% (w/w) fat (20.6% of the energy) supplied by one of four types of margarines. Margarines (Star S.p.A., Agrate Brianza, Italy) were prepared by interesterification of different proportions of sunflower seed oil, partially hydrogenated soybean oil, hydrogenated palm oil, and hydrogenated coconut butter. The LA content (percentage of FA) of the four margarines was 9.4, 14.9, 36.7 and 48.2%, respectively, corresponding to 1.9, 3.1, 7.7 and 10.1% of the energy provided by the diets. Diets were kept at 4°C in tightly closed plastic bags in a nitrogen atmosphere. To minimize oxidative alterations, feeds were replaced every day. The detailed composition of the diets is presented in Table 1.

TABLE 1

Fatty Acid Percent Composition of Fats in Experimental Diets[a]

Fatty acids	Diets			
	A	B	C	D
14:0	6.3	5.0	2.4	1.3
16:0	45.8	55.8	35.2	23.6
18:0	5.5	3.1	3.8	3.2
16:1	0.2	0.1	0.1	0.1
18:1	21.0	20.9	21.7	23.4
trans isomers	11.8	0.2	0.2	0.1
18:2n-6	9.4	14.9	36.7	48.2
18:2n-6 (as en%)	1.9	3.1	7.7	10.1

[a]All diets contained (en%): 17.4 protein, 20.9 fat, 61.7 carbohydrates, for a total of 430 Kcal/100 g diet.

At the end of the three months feeding period, animals were fasted overnight and killed after diethyl ether anesthesia. Blood was collected by intracardiac puncture using sodium citrate as anticoagulant. The following organs were removed and weighed: liver, heart, kidney, spleen, testicles and brain. The tissues were kept in small, air-tight containers at −20°C until analyzed.

Lipid extractions. Tissues were thawed, finely minced and 1-g aliquots of the homogeneous paste were homogenized in an Ultra Turrax (T-25 IKA, Staufen, Germany) tissue homogenizer, using chloroform/methanol (2:1, vol/vol) (11), containing 5 μg/μL of butyl hydroxytoluene for extraction. The lipid extract was concentrated under N_2 and the lipid concentration in aliquots was determined by the use of a Microbalance (C-31, Cahn Instruments, Cerritos, CA). Plasma lipids were extracted from diluted samples by stepwise addition of methanol and chloroform, and by collection of the organic layer after phase separation. Lipids were extracted from finely powdered diet samples following the same procedure as described for tissues.

Lipid analysis. Neutral (cholesteryl esters, triglycerides) and polar (total phospholipids [PL]) lipid classes in plasma lipid extracts were separated by thin-layer chromatography on prewashed silica gel (HR 60, Merck, Darmstadt, Germany) plates using hexane/diethyl ether/acetic acid (70:30:2, vol/vol/vol) as developing solvent. Compounds were detected on plates dried under N_2 by brief exposure to iodine vapors, the spots were scraped off, and the silica collected in vials for preparation of methyl esters.

Fatty acid methyl esters were prepared from total lipid extracts of the organs and from the separated lipid classes by transesterification with 3N methanolic HCl (Supelco, Bellefonte, PA). Methyl esters were analyzed on a Dani 8510 (Monza, Italy) gas chromatograph, equipped with a flame ionization detector, using chemically bonded Supelco Wax 10 (Supelco) column (30m, 0.32 mm i.d., 0.25 μm film) and temperature programming (140–210°C). Peaks were identified by comparison with pure reference compounds, and the percentage distribution of fatty acid methyl esters was quantified with a Shimadzu C-R 6A (Kyoto, Japan) recording integrator. The fatty acids were quantified relative to nonadecanoic acid (19:0) as internal standard, and calibration curves were obtained with reference compounds. The *trans* isomers present in margarine A and in lipid extracts from the same dietary group were separated by gas-liquid chromatography (GLC) analysis (data not reported).

Other assays. Glutamic-oxalacetic transaminase (GOT), lactic dehydrogenase (LDH) and glutamic-pyruvic transaminase (GPT), marker enzymes of liver function, were determined by standard assays. Plasma cholesterol and triglycerides (TG) were measured by enzymatic assays.

Statistical methods. The data for each tissue were submitted to one-way analysis of variance (ANOVA) and single comparisons between values at different levels of dietary LA were carried out according to Bonferoni *t*-test (12). Regression analysis was done for each tissue.

RESULTS

The diets used in this study were equicaloric and had an adequate composition (Table 1) of major and minor nutrients. The fat component consisted of one of four different margarines (A–D) with increasing levels of LA from 9.4 to 48.2 wt%; oleic acid contents, around 20%, were the same in all the diets, saturated acids ranged between 23.6 and 55.8%, with the lowest contents in the margarines which had the highest LA concentrations. Margarine A contained 11.8% *trans* isomers, mainly as elaidic acid.

Body growth, weekly weight gains, and caloric efficiencies [weight increments (g)/consumed diet (g)] (not shown) did not differ among groups. After three months of treatment, animals were sacrificed and organ weights, as percentage of body weights, plasma levels of cholesterol and TG levels of lipids in organs, and levels of enzymes markers of liver function, were determined. These parameters did not differ among groups.

The fatty acid composition of plasma, liver, heart and kidney total lipids were measured, but they are not reported here. In general, LA levels reflected those in the diet, but significant differences were observed for the long-chain PUFA in the various organs.

The relationships between LA intakes and the levels of n-6 and n-3 FA in plasma, liver and extrahepatic organs are shown in Figures 1–5. The values are expressed as μg/mg of plasma or tissue lipids and are superimposable with those expressed on a fresh weight basis, since levels of total lipids in tissues were not affected by dietary LA.

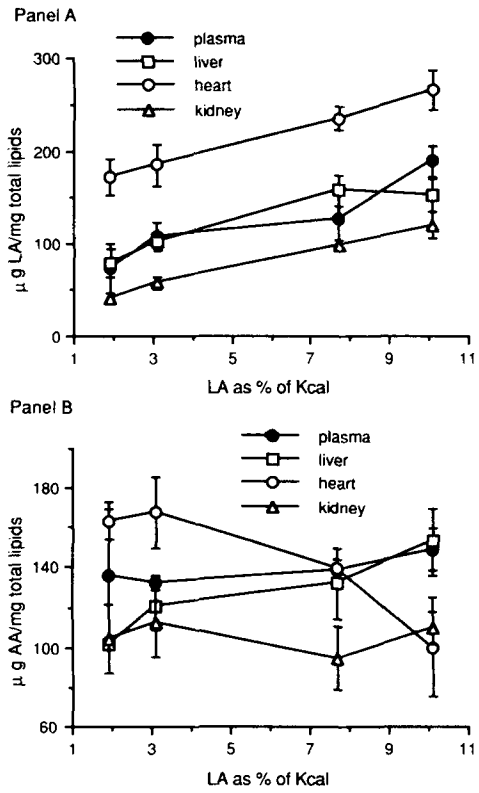

FIG. 1. Panel A. Levels (μg/mg of total lipids) of linoleic acid (LA) in plasma, liver, heart and kidney total lipids, with increasing levels of linoleic acid in the diet, as percentage of calories. Panel B. Levels (μg/mg of total lipids) of arachidonic acid (AA) in plasma, liver, heart and kidney total lipids, with increasing levels of linoleic acid in the diet, as percentage of calories.

The relationships between LA intakes and levels (μg/mg of total lipids) of LA and AA in plasma and different organs are shown in Figure 1. Changes of LA in all tissues (Panel A) followed similar trends, although the concentrations in the heart were almost twice those in the other tissues. Statistical analysis shows significant differences among values at different LA levels in the diet. One-way ANOVA shows statistically significant differences ($P < 0.05$ and $P < 0.01$) in all four tissues at different LA concentrations in the diet. The Bonferoni *t*-test shows, more specifically, that LA concentrations at the lowest dietary levels are always statistically different ($P < 0.05$) from those at the highest dietary levels. In liver, the LA concentration at the lowest dietary LA level is statistically different from the value at 7.7% LA in the diet. In all tissues, regression analysis of LA changes *vs.* increments in the diet indicate statistically significant linear correlations. The concentrations of AA in tissues (Panel B) varied according to different dietary LA levels; in the liver the concentration at 10 en% LA was significantly higher than at the lowest dietary LA levels, whereas in the heart AA concentration at 10 en% LA was significantly lower than at 1.9 and 3.1 en%. Regression analysis shows a statistically significant ($P < 0.05$) linear regression. In plasma and in the kidneys, there were no substantial changes, with increasing dietary LA.

When LA levels were measured in individual lipid classes of plasma and liver (Fig. 2), it appeared that LA accumulation in plasma (Panel A) occurred mainly in TG, up to a dietary LA concentration of 7.7 en%, whereas in liver (Panel B) LA increased mainly in PL, with a maximal concentration at 10 en% LA. Total AA in plasma (Fig. 3, Panel A) changed minimally with increasing LA in the diet, whereas in the liver (Panel B) AA concentration was significantly higher ($P < 0.0$) at 10 en% LA than at the lowest LA level in the diet.

Changes of n-3 FA in plasma and organs with increasing LA intakes also followed different trends. In plasma (Fig. 4, Panel A) both eicosapentaenoic acid (EPA) and DHA did not appreciably change up to 7.7 en% LA in the diet, but EPA rose with higher LA intake. In the liver (Panel B), EPA was marginally changed by different LA intakes, whereas DHA levels rose at 7.7 en% LA in the diet, and were very similar in the other groups. In the kidneys (Fig. 5) EPA and DHA levels remained constant, whereas in the heart EPA did not change, but DHA declined markedly and progressively, with a 50% reduction from the highest to the lowest concentrations. When DHA values were plotted against dietary LA, in the heart (not shown) a negative linear correlation, with a coefficient of 0.9, was obtained.

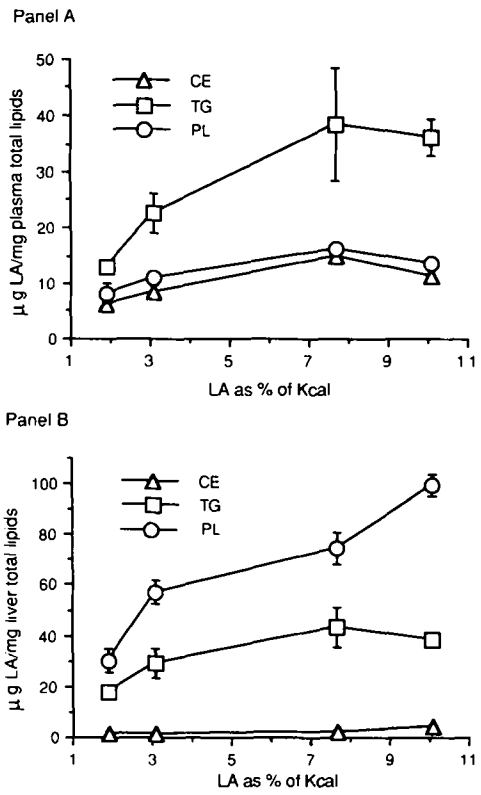

FIG. 2. Panel A. Levels (μg/mg of total liids) of linoleic acid (LA) in the major plasma lipid classes, with increasing levels of linoleic acid in the diet, as percentage of calories. Panel B. Levels (μg/mg of total lipids) of linoleic acid (LA) in the major lipid classes in the liver, with increasing levels of linoleic acid in the diet, as percentage of calories. CE, cholesteryl esters; TG, triglycerides; PL, phospholipids.

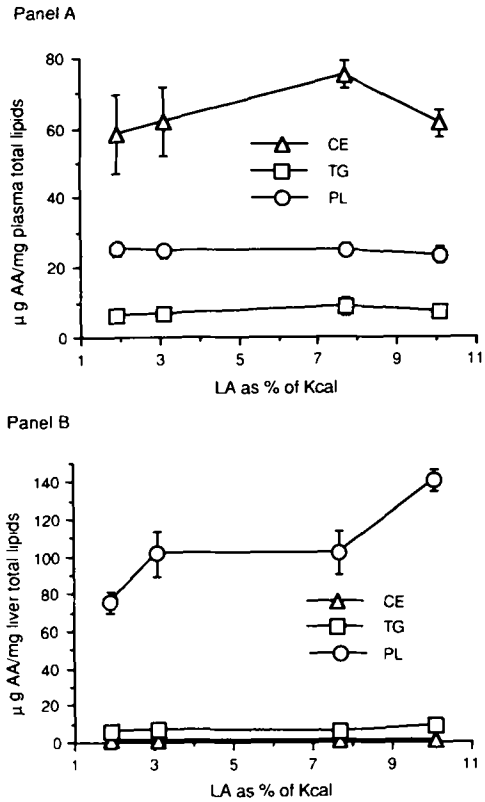

FIG. 3. Panel A. Levels (μg/mg of total lipids) of arachidonic acid (AA) in the major lipid classes in plasma, with increasing levels of linoleic acid in the diet, as percentage of calories. Panel B. Levels (μg/mg of total lipids) of arachidonic acid (AA) in the major lipid classes in the liver, with increasing levels of linoleic acid in the diet, as percentage of calories. CE, cholesteryl esters; TG, triglycerides; PL, phospholipids.

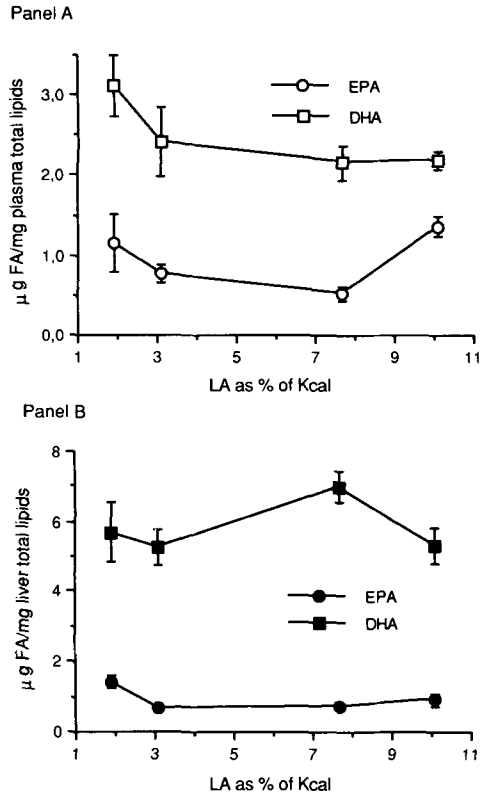

FIG. 4. Panel A. Levels (μg/mg of total lipids) of the n-3 fatty acids EPA and DHA in plasma total lipids, with increasing levels of linoleic acid in the diet, as percentage of calories. Panel B. Levels (μg/mg of total lipids) of the n-3 fatty acids EPA and DHA in liver total lipids, with increasing levels of linoleic acid in the diet, as percentage of calories.

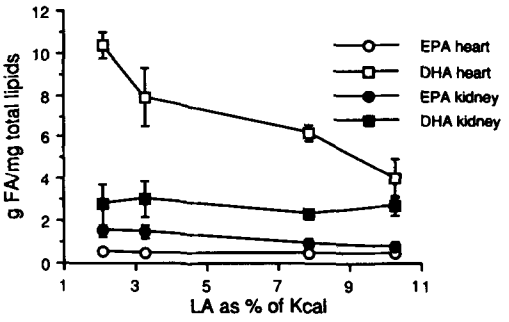

FIG. 5. Levels (μg/mg of total lipids) of the n-3 fatty acids EPA and DHA in heart and kidney total lipids, with increasing levels of linoleic acid in the diet, as percentage of calories.

DISCUSSION

During the last ten years, the effects of diets containing different types of fats and oils, and hence with different levels of LA, on plasma and tissue FA have been explored in a number of studies. The major conclusions that have generally been drawn are: i) the levels of PUFA in plasma and tissue lipids reflect the dietary intake, ii) dietary FA also influence the activity of the enzymes (especially desaturases) (13,14) in PUFA metabolism. Moreover, the direct contribution of preformed long-chain PUFA, such as AA (15) or the n-3 FA (16), present in the diet, to the endogenous pools of PUFA has only recently been appreciated. In the adult, the accumulation of AA in extrahepatic organs appears to be based mainly on processes such as delivery from the liver and uptake by cells, due to the very low activity of the desaturases and elongases in most tissues (17,18).

Very few studies have been directly devoted to investigating the effects of increasing levels of LA in the diet. In rodents it has been shown that the liver acts as a store of AA to be supplied to extrahepatic organs during essential fatty acid (EFA) deficiency (19,20); furthermore, when LA was increased to 10–11 en%, AA in various extrahepatic cells was reduced (21,22–24). Thus, in extrahepatic cells, remodelling of phospholipids (PL) through FA replacement, seems to prevail over the metabolic conversion of short-chain to long-chain PUFA, when LA is increased in the diet.

In our study when LA in the diet was raised from 2 to 10% of the energy, changes in LA, AA and n-3 FA in plasma, liver and organs such as heart and kidneys were distinctly different. In all tissues, increasing dietary LA resulted in a parallel increase in LA levels as expected. A detailed analysis of the changes of n-6 and n-3 PUFA in the different tissues revealed instead that elevation of AA, the major desaturation and elongation product of 18:2n-6, with increasing dietary LA, occurred only in the liver, mainly in the PL fraction, with reduction in the heart and practically no change in plasma and in the kidneys.

Our data, in agreement with Mohrhaurer and Holman (25), show that liver AA increased with increasing 18:2 in the diet, suggesting that there was no inhibition of LA conversion to AA. The lowering effect of dietary LA, at energy levels just above the minimal requirement, on heart AA has not been reported previously. Significant decline of both AA, at LA concentration in the diet greater than 3 en%, and of DHA, at LA concentration in the diet above 2 en% were observed in the heart. Similar effects were observed in previous studies using much higher concentrations of LA in the diet (23,24). In general, modifications of AA levels in heart lipids were also observed in animals fed different types of fats (26,27) but these studies have limited their comparisons to only a few scattered levels of dietary LA, and observed the reduction of long-chain PUFA in the heart only at very high amounts of dietary LA.

We have observed that in the heart the long-chain n-6 and n-3 FA were progressively replaced by a greater supply of dietary LA, the total amount of PUFA in heart structural lipids being held constant. Calculations of the total levels of n-6 and n-3 PUFA at 3 en% LA in the diet indicate that they are much higher in the heart (360 μg/mg lipid) than in the liver and the kidneys (228 and 173 μg/mg lipid, respectively). This observation and the findings of a reduction of long-chain PUFA in the heart with dietary LA levels above the minimum tested suggest that in the heart maximal PUFA levels are reached with dietary LA levels as low as 1.9 en%.

The accumulation of LA differentially affected the levels of n-3 FA in the liver and other organs. In the liver, there was no appreciable change in EPA and DHA levels, suggesting that the elevation of n-6 FA did not affect the n-3 fatty acid pool. The lack of decline of n-3 FA in the liver, in spite of the deficiency of n-3 in the diet, suggests that

under these conditions the liver was retaining n-3 FA and not supplying n-3 acids to extrahepatic organs. In the kidneys, n-3 levels, as well as those of 20:4n-6, were not appreciably modified by diet-induced accumulation of 18:2. In addition, we did not detect, in plasma and organs of the group fed the diet containing the *trans* FA isomers, an effect of these compounds on the levels of n-6 and n-3 PUFA.

The reduction of n-3 FA in the heart in addition to the fall of AA with increasing 18:2 in the diet may reflect inhibition of n-3 uptake by extrahepatic cells. This effect of high LA on n-3 levels in the heart could have significant consequences in regard to the recommendations for LA consumption. High LA intakes have been recommended for the prevention of atherosclerotic risk factors, but the reduction of DHA in the heart induced by high dietary 18:2 may bring about functional changes. In fact, a relationship between lower DHA levels in heart muscle and lower contraction force and greater sensitivity to arrhythmogenic agents have been reported (28,29).

In conclusion, our data show that when LA intake is increased there is an accumulation of AA only in the liver. In other organs, such as heart, the major changes are a decline in highly unsaturated FA of both the n-3 and n-6 series, and this in turn may differently influence AA-dependent processes in various organ.

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Incorporation of n-3 Fatty Acids of Fish Oil into Tissue and Serum Lipids of Ruminants

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This study examines the biohydrogenation and utilization of the C_{20} and C_{22} polyenoic fatty acids in ruminants. Eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids were not biohydrogenated to any significant extent by rumen microorganisms, whereas C_{18} polyenoic fatty acids were extensively hydrogenated. The feeding of protected fish oil increased the proportion of 20:5 from 1% to 13–18% and 22:6 from 2% to 7–9% in serum lipids and there were reductions in the proportion of stearic (18:0) and linoleic (18:2) acids. The proportion of 20:5 in muscle phospholipids (PL) increased from 1.5% to 14.7% and 22:6 from 1.0% to 4.2%; these acids were not incorporated into muscle or adipose tissue triacylglycerols (TAG). In the total PL of muscle, the incorporated 20:5 and 22:6 substituted primarily for oleic (18:1) and/or linoleic (18:2) acid, and there was no consistent change in the porportion of arachidonic (20:4) acid.
Lipids 27, 629–631 (1992).

The diets of grazing ruminants contain only 3–4% lipid, of which the galactosylglycerols are principal components and 18:3n-3 is the dominant fatty acid (1–3). For hand-fed ruminants, the principal feedstuffs are cereal grains, where the lipid content is also 3–4%, but 18:2n-6 is the major constituent fatty acid (3). Rumen microorganisms have evolved an effective capacity to hydrolyze complex lipids and hydrogenate the constituent C_{18} unsaturated fatty acids to *trans* monoenes and stearic acid (1). Ruminal hydrogenation can be prevented by encapsulating the oil in a matrix of aldehyde-treated protein (3) as has been done with sunflower oil resulting in significant incorporation of the constituent fatty acids, *e.g.* 18:2n-6 into the triacylglycerols (TAG) and phospholipids (PL) of ruminants (4).

The long-chain polyunsaturated fatty acids of fish oil (20:5 and 22:6) are known to be effective in reducing the risk of coronary heart disease as they are antithrombotic (5) and antiarrhythmic (6). For this reason it has been suggested that it might prove useful to introduce n-3 C_{20} and C_{22} into the tissues of meat animals (7). This study examines the biohydrogenation and utilization of the C_{20} and C_{22} polyenoic fatty acids in ruminants.

METHODS

Animals and diets. Both sheep and cattle were used in the experiments. The sheep used were 18–24-month-old Merino wethers of approximately 35 kg body weight. The cattle used were 12–18-month-old European × British crossbred steers of approximately 300 kg body weight. Both species were fed a basal diet composed (w/w) of the following: 30% wheaten chaff, 30% maize, 20% lupin, 15% wheat screenings, 2.5% calcium carbonate and 2.5% sodium chloride. This diet was supplemented with fish oil ("MAXEPA", Scherer Pty Ltd, Melbourne, Australia), which had been encapsulated in a matrix of formaldehyde-treated protein to prevent ruminal hydrogenation (8) and loss of appetite that occurs in ruminants when the amount of dietary fat exceeds 2 to 3% (3). The protected fish oil (PFO) supplement contained 40% fat and had the following major fatty acid composition: 16:0, 18.2%; 18:1, 12.4%; 20:5, 17.6%; 22:6, 11.5%.

Two sheep were fed the basal ration and four sheep were supplemented with PFO and fed at 20% and 30% by weight of the diet. Animals were introduced to the diets over a period of one week with the total amount of treated supplement being gradually increased during that time. One steer received the basal ration and two were supplemented with PFO. Samples (100 mg) of muscle (*Longissimus dorsi*) were removed from the cattle by biopsy technique prior to offering the supplements and at weekly intervals for four weeks. Both the sheep and cattle were slaughtered after five weeks of feeding and muscle and adipose tissues (subcutaneous) were sampled and frozen prior to fatty acid analysis.

Analytical methods. Biohydrogenation and lipolysis of fish oil and sunflower oil supplements were assessed using procedures described previously (8). Blood samples were taken by jugular vein puncture; serum was separated and fatty acid analysis performed using procedures described elsewhere (4). In brief, muscle and adipose tissue lipids were extracted into chloroform/methanol (2:1, vol/vol) containing 0.01% (w/v) butylated hydroxytoluene. Neutral lipids and phospholipids (PL) of muscle were separated by thin-layer chromatography (TLC) on silica gel G plates (200 × 200 × 0.25 mm) using hexane/diethyl ether/acetic acid, 70:30:1 (vol/vol/vol). Individual PL were separated by TLC using procedures previously described (9). Areas of gel corresponding to the triacylglycerols (TAG) and PL were scraped off the plate and the lipid extracted (10). Fatty acid methyl esters were prepared (11) and analyzed by gas chromatography (Varian model 3400, Palo Alto, CA) using a 25-m × 0.25-mm fused silica capillary column (BPX70, SGE Pty Ltd, Melbourne, Australia). Fatty acids were identified by comparison of retention times with standard fatty acid methyl ester (FAME) mixtures.

RESULTS

Comparison of lipolysis and biohydrogenation of fish oil and sunflower oil by rumen microorganisms. Incubation of freeze-dried preparations (not treated with aldehyde) of fish oil-casein (1:1, w/w) and sunflower oil-casein (1:1, w/w) with rumen microorganisms for 24 h under anaerobic conditions resulted in complete lipolysis of TAG to free fatty acids (Fig. 1). Despite this, negligible hydrogenation of the n-3 C_{20} and C_{22} fatty acids could be demonstrated

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Abbreviations: CE, cholesteryl esters; DAG, diacylglycerols; FAME, fatty acid methyl esters; FFA, free fatty acids; PFO, protected fish oil; PL, phospholipids; TAG, triacylglycerols; TLC, thin-layer chromatography.

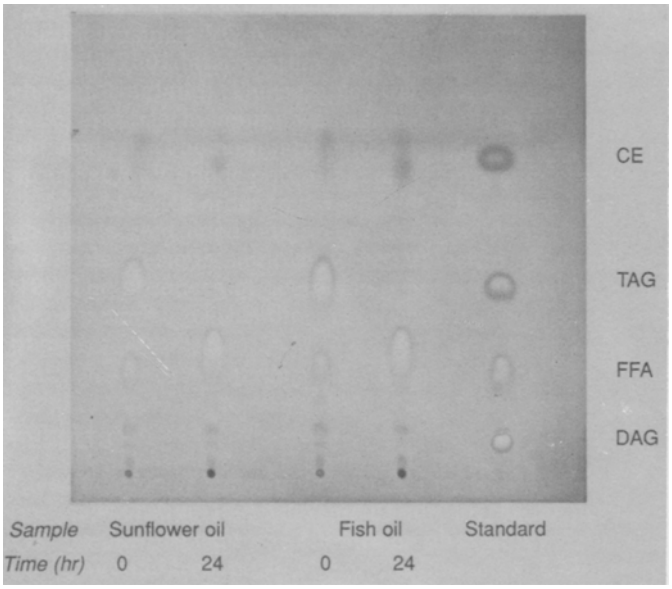

FIG. 1. One-dimensional TLC separation of lipid extracts from rumen incubations. Rumen contents from sheep (10 mL) were anaerobically incubated for 24 h at 37°C with freeze-dried preparations of sunflower oil-casein (1:1, w/w) and fish oil-casein (1:1, w/w), 50 mg, not protected with aldehyde (9). Individual lipids, *i.e.* cholesteryl esters (CE), triacylglycerols (TAG), free fatty acids (FFA) and diacylglycerols (DAG) were visualized by spraying with 2,7-dichlorofluoroscein.

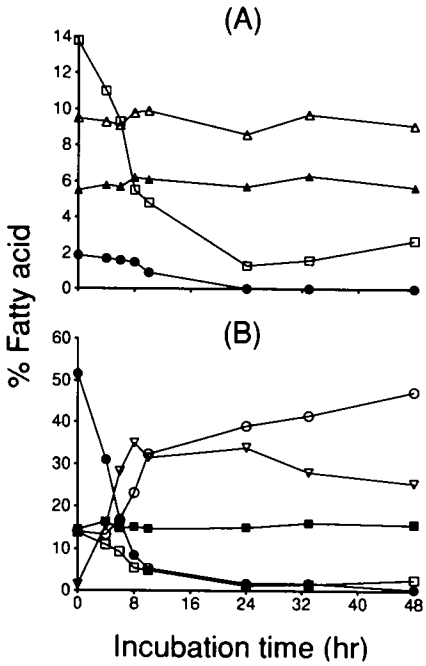

FIG. 2. Comparison of the hydrogenation of C_{18}, C_{20} and C_{22} unsaturated fatty acids by rumen microorganisms. Freeze-dried preparations (100 mg) of (A) fish oil-casein (1:1, w/w) and (B) sunflower oil-casein (1:1, w/w), not treated with aldehyde, were anaerobically incubated with sheep rumen contents at 37°C. Fatty acids were analyzed as described in the text. In (A), 16:0 and 18:0 are not shown and account for 45% of the total fatty acids. (■) 16:0, (○) 18:0, (□) 18:1 *cis*, (▽) 18:1 *trans*, (●) 18:2, (△) 20:5 and (▲) 22:6.

when these fish oil preparations were incubated with strained rumen liquor obtained from sheep, for up to 48 h under anaerobic conditions (Fig. 2a), whereas the 18:1 and 18:2 present in the fish oil were hydrogenated by rumen microorganisms as demonstrated by the increase in the intermediary 18:1 *trans* (2.6% to 4.8%) and an increase in 18:0 (12.7% to 16.3%, data not shown). For comparison Figure 2b shows the biohydrogenation of C_{18} unsaturated fatty acids of sunflower oil with 18:1 *trans* and 18:0 being the principal end-products.

The extent of hydrogenation of 20:5 and 22:6 was assessed by incubating the freeze-dried preparations of fish oil-casein (not treated with aldehyde) with rumen microorganisms removed from the sheep 30 days after the start of fish oil supplementation. During a 24-h incubation under anaerobic conditions, the proportions of 20:5 and 22:6 in the total rumen incubation contents were virtually unchanged, *viz.* 13% *versus* 13.3% and 7.9% *versus* 7.0% for 20:5 and 22:6, respectively.

Fatty acid composition. The composition of the major fatty acids in serum lipids of sheep is illustrated in Table 1; inclusion in the diet of the fish oil supplement increased the proportion of 20:5 and 22:6. For example the 20:5 content of serum lipids increased from 1 to 13% after feeding 20% fish oil supplement and reached 18% in the sheep receiving 30% fish oil supplement (Table 1). Similarly the proportion of 22:6 increased in response to the level of supplementation and the total n-3 serum fatty acids increased 5- to 7-fold (Table 1). The proportion of 20:4n-6 was unchanged and there were decreases in the proportion of 18:0 and 18:2 (Table 1).

Analysis of the fatty acid composition of adipose tissue from sheep fed the fish oil supplements revealed that 20:5 or 22:6 was not detectable (Table 1). The proportion of 20:5 and 22:6 increased 3–4-fold in the total muscle PL of sheep (Table 1) and cattle as a result of including the PFO in the diet. In sheep, the mean values of the proportion of 20:5 for the 20% and 30% supplementation were not significantly different, but when these values were pooled the mean of supplemented animals (11.0%) were significantly different ($P < 0.05$) to that of unsupplemented animals (2.4%). In individual cattle, the values of 20:5 increased at similar rates over the 5-week period, reaching 15.6% and 16.7% for 20% and 30% supplementation, respectively, from a base of 4.1%. In sheep, there were reductions in the proportion of 18:1 and no consistent change in 20:4.

Analysis of the choline PL of muscle from sheep fed PFO clearly indicated that the proportion of 20:5 increased and there was a reduction in the content of 18:1. The 20:5 content rose to a mean value of 11.4% when 20% fish oil supplement was fed in the diet to two animals and to a mean of 15.4% when 30% was fed. The 18:1 value fell to 18.1% from 24.6% when 30% was fed.

DISCUSSION

The study demonstrated that the C_{20} and C_{22} fatty acids of fish oil, even when not protected, are not hydrogenated to any significant extent. This is in contrast to oils containing C_{18} fatty acids where extensive hydrogenation occurs (1–3). Furthermore hydrogenation of the C_{20} and C_{22} fatty acids could not be detected in rumen microbial lipids of sheep, even after feeding fish oil supplements for 30 d. The inability of rumen microorganisms to hydrogenate

TABLE 1

Effect of Feeding PFO on the Fatty Acid Composition of Sheep

Fatty acid	Control		PFO[a] (20%)		PFO[a] (30%)	
	242	261	390	253	396	463
Serum total lipids						
16:0	22.7	19.5	16.1	18.7	15.7	20.6
18:0	25.4	24.4	9.3	10.7	9.2	10.5
18:1n-9	15.9	17.4	26.3	22.7	21.5	18.3
18:2n-6	19.5	17.5	9.0	8.9	7.4	6.3
18:3n-3	0.4	0.4	0.4	0.4	0.2	0.2
20:4n-6	3.4	4.5	4.0	4.3	4.4	5.9
20:5n-3	0.8	1.0	13.7	13.7	18.3	17.4
22:5n-3	1.0	1.7	3.4	2.7	4.2	3.1
22:6n-3	1.5	2.1	6.9	7.0	8.9	7.7
Σ n-3	3.7	5.2	24.4	23.8	31.6	28.4
Σ n-6	22.9	22.0	13.0	13.2	11.8	12.2
Subcutaneous adipose tissue						
16:0	21.9	20.1	19.9	21.6	20.9	22.5
18:0	26.2	31.8	27.2	25.0	22.0	24.8
18:1n-9	39.2	38.8	38.0	37.9	37.8	39.1
18:2n-6	2.5	1.9	2.7	2.3	1.8	2.1
18:3n-3	1.2	1.3	0.9	0.8	2.3	2.0
20:5n-3, 22:5n-3, 22:6n-3	n.d.[b]	n.d.	n.d.	n.d.	n.d.	n.d.
Muscle TAG						
16:0	24.2	23.9	22.0	19.8	25.1	22.8
18:0	21.1	16.0	23.2	23.2	16.6	16.6
18:1n-9	41.4	43.3	43.2	43.6	41.6	44.9
18:2n-6	3.2	2.1	2.5	3.2	2.3	2.3
18:3n-3	1.5	1.2	0.7	1.7	1.1	0.7
20:5n-3, 22:6n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Muscle PL						
16:0	14.2	15.2	11.6	9.9	16.8	16.1
18:0	11.4	11.6	10.4	8.3	13.4	11.3
18:1n-9	21.1	21.0	17.2	14.8	17.4	14.3
18:2n-6	24.9	23.0	27.3	26.5	17.8	16.4
18:3n-3	2.2	2.2	2.2	2.7	0.6	0.6
20:4n-6	9.7	11.1	12.6	12.1	9.2	9.6
20:5n-3	1.5	3.3	9.8	11.0	8.5	14.7
22:5n-3	2.1	2.7	2.6	2.9	2.7	1.2
22:6n-3	1.0	1.4	2.9	4.2	4.1	3.6
Σ n-3	6.8	9.6	17.5	20.8	15.9	20.1
Σ n-6	34.6	34.1	39.9	38.6	27.0	26.0

[a]Sheep were fed PFO as 20% and 30% by weight of the diet for 35 days.
[b]n.d., not detectable.

the 20:5 and 22:6 compared to the C_{18} fatty acids is not the result of the fish oil inhibiting lipase activity in the rumen, because the degree of lipolysis of sunflower oil and fish oil was similar, and is therefore probably due to the absence of specific enzymes or steric factors.

The feeding of PFO increased the proportion of 20:5 and 22:6 in the serum total lipids with corresponding reductions in the proportions of 18:0 and 18:2. Increases of 20:5 and 22:6 occurred in sheep and cattle muscle PL, but not in adipose tissue or muscle TAG. In previous studies, feeding or infusing cod liver oil in an unprotected form increased the proportion of C_{20} and C_{22} unsaturated fatty acids in serum and milk (12–14). Therefore it appears that ruminants are able to incorporate n-3 C_{20} and C_{22} fatty acids of fish oil into milk fat, but not into TAG of adipose tissue or muscle. It is clear that ruminants are able to preferentially incorporate 20:5 and 22:6 into PL and do so by substitution of the 18:1 and/or 18:2 fatty acids. This pattern of substitution is similar to that observed in rats (15) and marmoset monkeys (16) and indicates a preferential incorporation of n-3 over n-6 fatty acids. However the proportion of 20:4 is unchanged in both serum and muscle PL, which would suggest that the Δ6 desaturase activity was not inhibited by the elevated 20:5 content. This contrasts with the finding of Garg *et al.* (15), who showed that the feeding of a fish oil and hydrogenated tallow supplement to rats increased the 20:5 content of serum total lipids to 16% and reduced the 20:4 content from 15% to 6%.

In summary, the n-3 C_{20} and C_{22} fatty acids of fish oil are not hydrogenated in the rumen but are incorporated into muscle PL. However, because the PL fraction represents only a small proportion of the fat in ruminant meat, it does not appear to offer a means of providing humans with a significant source of these fatty acids.

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Comparison of Sulfoquinovosyl Diacylglycerol from Spinach and the Purple Bacterium *Rhodobacter sphaeroides* by Fast Atom Bombardment Tandem Mass Spectrometry

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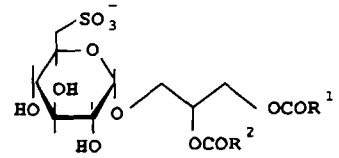
Isolated sulfoquinovosyl-diacylglycerol (SQD) from spinach and the purple bacterium *Rhodobacter sphaeroides* provide two sources of very different molecular species of SQD. We were able to demonstrate by fast atom bombardment-collisionally activated dissociation tandem mass spectrometry in the negative ion mode that the sulfoquinovosyl head group of the plant and bacterial lipids can be characterized by the common fragmentation pattern found in the spectra of both samples. Differences in the acyl functions from the two sources were also identified by this technique. SQD specific fragments are found at *m/z* 299, 283, 241, 225, 165 and 80 which indicate the presence of the sulfoquinovosyl moiety. The two predominant molecular species found in spinach contain palmitic and linolenic ($[M-H]^-$ at *m/z* 815) or two linolenic acids ($[M-H]^-$ at *m/z* 837) in the *sn*-1 and *sn*-2 positions, while the two major species of the bacterial lipid contain palmitic and 18:1 (vaccenic) acids ($[M-H]^-$ at *m/z* 819) or stearic and 18:1 (vaccenic) acids, ($[M-H]^-$ at *m/z* 847), respectively.
Lipids 27, 632-636 (1992).

Approximately thirty years ago, Benson and co-workers (1) discovered that $^{35}SO_4$-labeled extracts from various higher plants, algae, and a purple non-sulfur bacterium harbored significant amounts of a sulfur containing lipid. They determined the structure to be 1-*O*-(*α*-6′,6-deoxy-aldohexopyranosyl-6′-sulfonic acid)-3-*O*-diacylglycerol, **1** (2). The 6-deoxy-6-sulfo-glycose is also known as sulfoquinovose. The two major fatty acids found in the sulfoquinovosyl diacylglycerol (SQD) in higher plants are palmitic acid and linolenic acid (3). In SQD of *Rhodobacter sphaeroides*, a purple non-sulfur bacterium, the place of linolenic acid is taken by vaccenic acid (18:1, *cis* Δ11), a monounsaturated 18-carbon fatty acid commonly found in bacteria (4). Positional analysis of the fatty acyl groups of SQD in higher plants had previously revealed an interesting difference compared to the galactolipids monogalatosyl diacylglycerol (MGD) and digalactosyl diacylglycerol (DGD) (3). Chloroplastic lipids such as MGD and DGD contain palmitic acid preferentially in the *sn*-2 position and linolenic acid in the *sn*-1 position, whereas SQD contains palmitic acid in the *sn*-2 as well as in the *sn*-1 position of the glycerol backbone (3).



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SQD occurs in all higher plants, including ferns and mosses and in various classes of algae such as hemoflagellata, phytoflagellata, red algae, brown algae and green algae. SQD has also been reported to be a constituent of cyanobacteria and some species of purple non-sulfur bacteria, including *R. sphaeroides* (5). The amounts, measured in percent of ether extractable lipids, vary from 2.6% in *R. sphaeroides* up to 18.3% in the brown algae *F. vesiculosa* (5). Leaves of higher plants usually contain around 5% SQD of the extractable lipids (6). A large amount of organic sulfur exists as SQD, which therefore plays an important role in the global sulfur cycle (7). The exclusive occurrence of SQD in photosynthetic organisms, its subcellular localization in photosynthetic membranes, and the unique structure of the sulfonic acid head group, indicated early on that SQD may play a critical role in photosynthesis. Indirect evidence has been accumulated which supports this idea (8,9).

To date, few direct methods for the analysis of sulfoquinovosyl-diacylglycerols have been reported. An early study by Budzikiewicz and co-workers (10) employed electron impact mass spectrometry (EIMS) to analyze the permethylated derivative of SQD, but the spectra contain a very weak molecular ion (~0.1% relative abundance). A single fragment indicative of the sulfoquinovosyl moiety is present at *m/z* 367, but other ions only permit the total weight of the two acyl groups to be determined. By this approach, it is not possible to characterize the two acyl groups or their relative positions. Here we report the use of fast atom bombardment-mass spectrometry (FAB-MS) and fast atom bombardment-collisionally activated dissociation-tandom mass spectrometry (FAB-CAD-MS/MS) in the negative ion mode as diagnostic methods to analyze mixtures of SQD molecular species and to identify the presence of the sulfoquinovosyl head group in lipids from two different sources. In addition, we demonstrate that FAB-CAD-MS/MS can be directly used to characterize the composition of the two acyl groups and possibly their relative positions.

MATERIALS AND METHODS

Preparation of SQD. Spinach leaves (500 g) obtained from a local store were blended in 1.2 L of chloroform/methanol/water (5:5:1, vol/vol/vol). Solids were separated by filtration and re-extracted with 440 mL chloroform/methanol/H_2O (5:5:1, vol/vol/vol). The two combined filtrates were phase partitioned with 600 mL 1M KCl, 0.2M phosphoric acid. The dried chloroform phase yielded about

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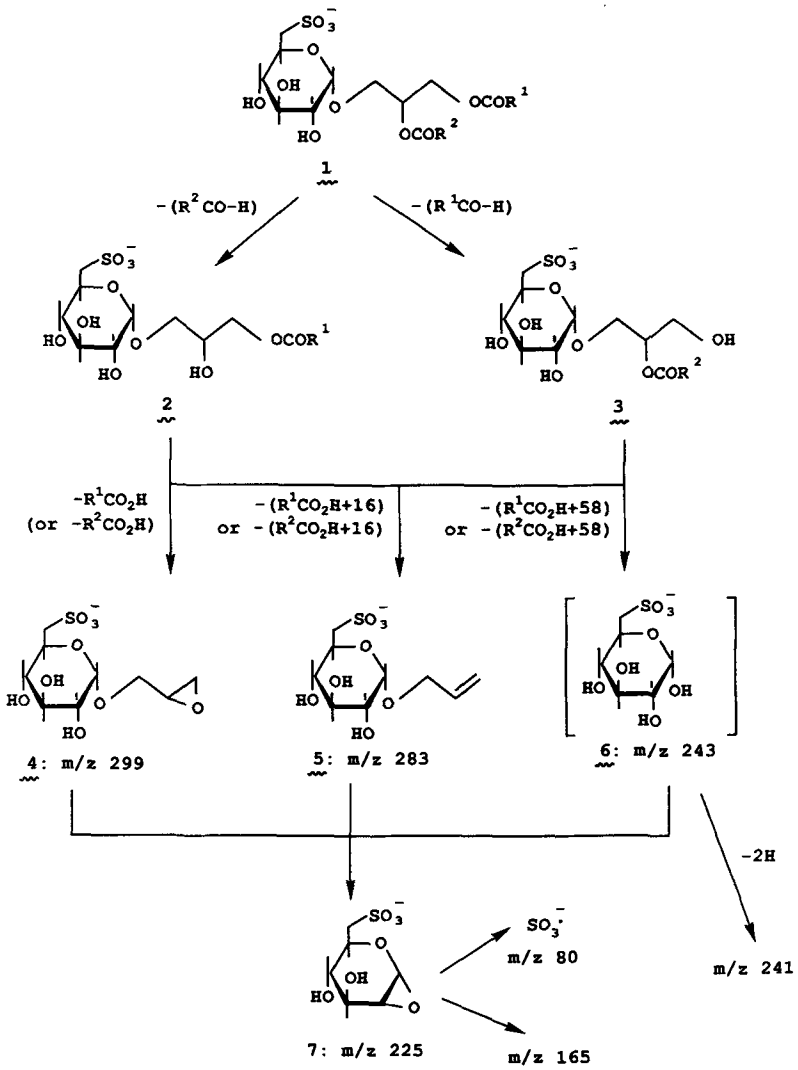
Abbreviations: B/E, ratio of magnetic sector field strength to electrostatic analyzer energy; DGD, digalactosyl diacylglycerol; EIMS, electron impact mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; FAB-CAD-MS/MS, fast atom bombardment-collisionally activated dissociation-tandem mass spectrometry; GC, gas chromatography; MGD, monogalactosyl diacylglycerol; MS/MS, tandem mass spectrometry; *m/z*, mass to charge ratio; SQD, sulfoquinovosyl diacylglycerol; TLC, thin-layer chromatography.

4.5 g of lipids. This material was purified according to O'Brien and Benson (11). Similarly, SQD was extracted and purified from 20 g of *R. sphaeroides* (2.4.1) cells grown photoheterotrophically in Sistrom's medium (12), except that lipid extraction volumes were cut in half. As a final purification step, thin-layer chromatography (TLC) was employed to remove contaminating phosphatidylglycerol. Baker Si 250 silica gel plates were first soaked in 0.15 M ammonium sulfate. Following complete drying in air, the plates were activated for 2.5 h at 120°C and after application of lipid extract, developed in acetone/benzene/water (91:30:8, vol/vol/vol). The SQD band was scraped off the plate after visualizing with iodine vapor and the lipid was eluted using chloroform/methanol (1:1, vol/vol). In addition, this TLC system was used to monitor the purity of SQD throughout the purification procedure.

Mass spectrometry. Approximately 1.0 μg of the sulfolipid sample dissolved in chloroform/methanol (1:1, vol/vol) was mixed with 2 μL of triethanolamine matrix on the FAB probe tip. Ions were produced by bombardment with a beam of Xe atoms (6 keV) or Cs^+ ions (12 keV) in a JEOL HX-110 double focusing mass spectrometer (JEOL USA, Peabody, MA) operating in the negative ion mode; no differences were observed in spectra acquired by neutral beam (Xe°) or charged cesium beam (Cs^+) bombardment. The accelerating voltage was 10 kV and the resolution was set at 1000. For FAB-CAD-MS/MS, helium was used as the collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the abundance of the parent ion by 50%. A JEOL DA-5000 data system generated linked scans at a constant ratio of magnetic to electrical fields (B/E). The instrument was scanned in two minutes from *m/z* 0–1500. Data presented were acquired in a single scan, although data acquired on subsequent scans were found not to differ.

RESULTS AND DISCUSSION

Comparison of molecular species of sulfolipid found in samples of spinach and R. sphaeroides. The negative FAB spectra of the two sulfolipid samples obtained from spinach and *R. sphaeroides* displayed prominent peaks for the $[M-H]^-$ ions (1, Scheme 1) of the individual molecular species present (Fig. 1). Two predominant molecular



SCHEME 1

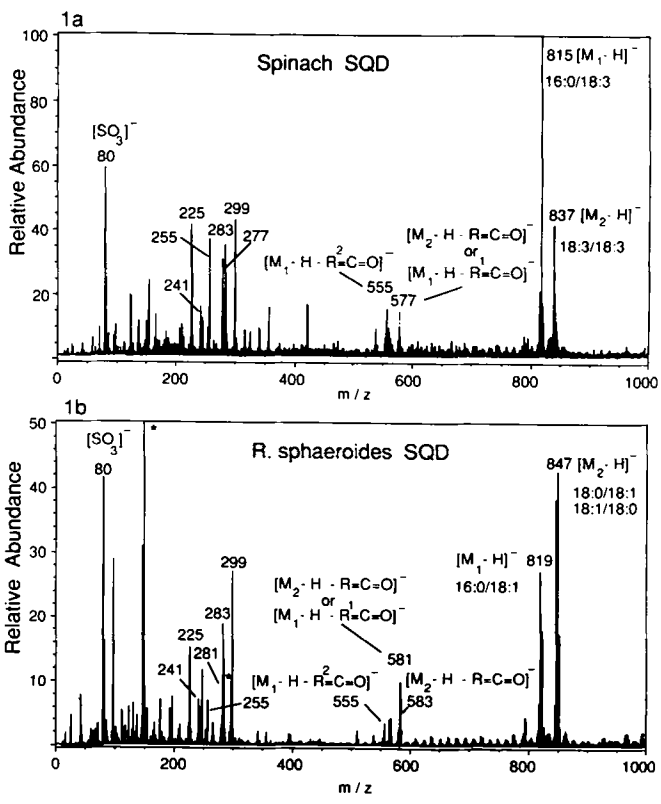


FIG. 1. The negative FAB mass spectra of spinach SQD (Fig. 1a) and *R. sphaeroides* SQD (Fig. 1b). Each spectrum dislays $[M-H]^-$ ions representing the molecular species present. The two predominant molecular species in each spectrum are designated by the nomenclature referring to the acyl group positions, *sn*-1/*sn*-2 (minor species are discussed in the text). Confirmation of the assignment of particular acyl groups to a particular molecular species in a mixed sample, however, requires MS/MS analysis of the individual $[M-H]^-$ ions (see Fig. 2). Other fragments in the spectra at *m/z* 80, 225, 241, 283 and 299 are characteristic of the sulfoquinovosyl glycerol moiety. Triethanolamine matrix ions at *m/z* 148 and 297 are designated with an asterisk in Figure 1b. Because of the higher concentration of SQD in the spinach sample (Fig. 1a), these matrix ions are suppressed.

species were found in the spinach sample at *m/z* 815 and 837 (Fig. 1a), which were interpreted as sulfoquinovosyl diacylglycerol containing two acyl functions corresponding to a total fatty acid composition (carbon atoms and double bonds) of 34:3 and 36:6, respectively. The $[M-H]^-$ ions of minor molecular species were also observed at *m/z* 813, 817 and 839; these likely differed from the major molecular species in the degree of acyl group unsaturation. This interpretation was consistent with previously reported data obtained by conventional gas chromatography (GC) analysis of the fatty acid composition of spinach sulfolipid, where palmitic and linolenic acids were the main acids detected (38.9 and 45% of the total, respectively) and linoleic, 16:1, oleic, 16:3 and stearic acids were minor components (6.1, 5.4, 1.9, 1.5 and 1.2%, respectively) (13,14). Corresponding fragments representing the carboxylate anions ($RCOO^-$) of these fatty acyl groups in the low mass region of the FAB spectrum (*e.g*, major ions at *m/z* 255 and 277 for 16:0 and 18:3, respectively) supported the GC data. However, note that if a sample is contaminated with free fatty acids, these low mass ions may be misleading. Fragments in the spectrum were also found at *m/z* 555 and 577 as a result of the loss of one fatty acyl group in the form of its ketene analog (R=C=O) (2 and 3, Scheme 1) suggesting the presence of an 18:3 acyl group in both molecular species ($[M-H-260]^-$ in Fig. 1a). The negative FAB spectrum of bacterial sulfolipid showed two principal species of sulfoquinovosyl diacylglycerol with $[M-H]^-$ ions at *m/z* 819 and 847 (Fig. 1b), which correspond to two molecular species containing either fatty acyl groups totalling 34:1 or 36:1. The presence of other molecular species was indicated by ions at *m/z* 817 (34:2), 821 (34:0), 845 (36:2), and 849 (36:0). The fatty acid composition obtained by GC analysis of *R. sphaeroides* SQD indicated palmitic, stearic and vaccenic acids are the primary acyl groups present (8). The later compound, typical of bacteria, is an eighteen carbon *cis*-Δ11 acid. Again, carboxylate anions in the FAB spectrum at *m/z* 255, 281 and 283 lend support to the GC data and the interpretation of the molecular ion clusters. As in the previous spectrum, loss of a fatty acyl moiety (as the ketene analog, R=C=O) resulted in the formation of ions at *m/z* 555, 581 and 583 (2 and 3, Scheme 1) which are consistent with the presence of palmitoyl, stearoyl and vaccenoyl (or an 18:1 isomer) acyl groups (loss of 238, 266 or 264, respectively from the $[M-H]^-$ ions.). In addition, the spectra of both the spinach and bacterial sulfolipid both showed common fragment peaks at *m/z* 299, 283, 241, 225 and 80, which were suspected to be characteristic of SQD.

FAB-CAD-MS/MS analysis of molecular ions and high mass fragments of sulfolipid. In order to further characterize the molecular species in the two samples, two B/E linked scans of the $[M-H]^-$ ions of the major species found in the spinach sample (*m/z* 815) and the bacterial sample (*m/z* 847) were obtained (Fig. 2). Collisionally activated dissociation induced the formation of a number of fragments between *m/z* 600–800 at intervals of about 14 mass units. These correspond to fragmentation along the fatty acyl chains and represent charge-remote fragmentation (15) similar to that reported in the CAD-MS/MS spectra of phospholipids (16). Although in principle these fragments could be used to locate sites of unsaturation (15), the contribution of overlapping fragments from both acyl groups makes interpretation problematic. In each spectrum, two prominent fragments are observed in the middle mass range which result from loss of the acyl groups as their corresponding free acids (−RCOOH). These fragments confirm the identities of the two acyl groups in the spinach SQD species as palmitoyl and linolenoyl and in the main *R. sphaeroides* SQD molecular species as palmitoyl and vaccenoyl (or an 18:1 isomer). The relative intensity of these two fragments may provide evidence for the relative positions of the two acyl functions. By analogy to phosphatidylcholine, loss of the *sn*-2 acyl group is expected to be favored (17), thus yielding a more abundant $[M-H-R^2COOH]^-$ fragment (see Fig. 2). However, in the case of these SQD samples, we have no independent determination of the acyl group positions (but see 14). In the low mass range, fragments characteristic for the sulfoquinovosyl glycerol group were found. Both spectra showed a similar pattern of fragments with ions at *m/z* 299 (weak), 283, 241, 225, and 80 (weak). These identical loss mass daughter ions were detected when B/E linked scans were performed on precursor ions at *m/z* 555 (Fig. 3a) and 577 for spinach and on those at *m/z* 581 (Fig. 3b) and 555 for the bacterial sample, which represent

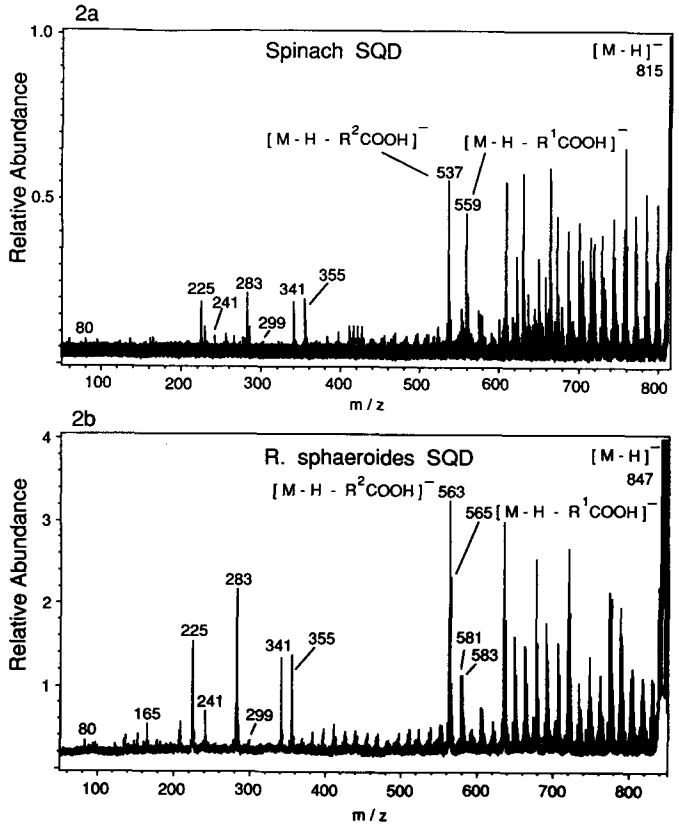


FIG. 2. FAB-CAD-MS/MS of the prominent $[M-H]^-$ ions representing the most abundant SQD molecular species in spinach (Fig. 2a) and *R. sphaeroides* (Fig. 2b). The precursor ion at *m/z* 815 in Figure 2a yields fragments in the high mass end of the spectrum from remote site fragmentation (15) along the acyl group chains. Two prominent daughter ions at *m/z* 537 and 559 result from loss of a linolenoyl and a palmitoyl group, respectively, as their corresponding free acids (RCOOH). Ketene losses are much less favored under these experimental conditions (cf. Fig. 1). Low mass fragments from the $[M-H]^-$ ion at *m/z* 847 in the bacterial SQD sample (Fig. 2b) are identical to those observed in the spectrum of the SQD molecular species from spinach (Fig. 2a). The daughter ions at *m/z* 563 and 565 show that the acyl groups in this case are stearoyl and vaccenoyl (or another 18:1 isomer).

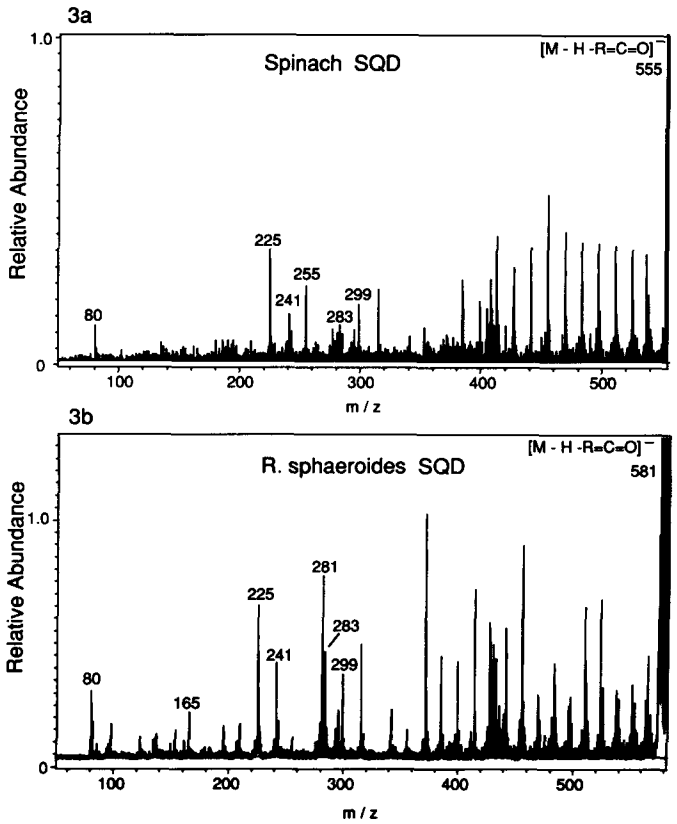


FIG. 3. FAB-CAD-MS/MS of the $[M-H-R=C=O]^-$ ions at *m/z* 555 in the spinach sample (Fig. 3a), representing monoacyl SQD with a palmitoyl group and at *m/z* 581 in the *R. sphaeroides* sample (Fig. 3b), representing monoacyl SQD containing a vaccenoyl function. Fragments are observed in both spectra at *m/z* 80, 225, 241, 283 and 299 which are characteristic of sulfoquinovosylglycerol (see Scheme 1). In addition, the more concentrated *R. sphaeroides* sample displays another diagnostic peak at *m/z* 165. The spectrum of each sample contains a peak for the carboxylate anion of their respective acyl groups at *m/z* 255 (palmitate) in Figure 3a and at *m/z* 281 (vaccenate) in Figure 3b. Other fragments in the high mass region are formed by remote site fragmentation along the acyl chain (15,16).

the respective molecular species following the loss of one fatty acyl group as the ketene analog (2 and 3, Scheme 1). The appearance of the same fragments in all these spectra lends support to the notion that these ions are related to the sulfoquinovosyl glycerol moiety, rather than to the acyl groups. The structure and formation of these fragments were further investigated by additional FAB-CAD-MS/MS experiments described below.

FAB-CAD-MS/MS of sulfolipid specific fragments. In any SQD, loss from the $[M-H]^-$ ion of one acyl group as the free acid and the second acyl group as its ketene analog should yield an ion of mass 299. This could be represented as the ion 4 in Scheme 1. The B/E linked scan of the ion at *m/z* 299 in the negative FAB spectrum of the spinach sample showed fragments at *m/z* 225, 165, and 80. Consistent with the hypothetical structure of the ion at *m/z* 299, the ion at *m/z* 225 likely represents the epoxysulfoquinovosyl fragment (7, Scheme 1) formed by the loss of the deoxyglycerol moiety in 4 (m/z 299), while *m/z* 80 represents the sulfonyl anion. The absence of a fragment at *m/z* 241 suggested that this ion was derived from the molecular ion or the products resulting from the loss of one acyl group, but that its direct formation from fragment at *m/z* 299 was not favored. The proposed formation of the observed fragments of the ion at *m/z* 299, 4 is shown in Scheme 1. A structure for the ion at *m/z* 165 could not be proposed. This latter ion was not observed in the B/E linked scan of the ion at *m/z* 241 (see below). Analysis of the ion at *m/z* 283, the deoxy analog of 4, provided similar results, yielding major fragments at *m/z* 225, 165 and 80. The proposed structure for this ion, 5, is shown in Scheme 1. The absence of stearic acid in the fatty acid analysis excludes the possibility that this peak represents the stearic carboxylate anion. Further, the daughter ion spectrum of this ion is quite distinct from that of stearate.

The sulfoquinovose sugar anion has a mass of 243. Consistently in all the spectra discussed above, a key ion was observed at *m/z* 241, although the ion at *m/z* 243 was present in less abundance (Fig. 1). It is proposed that the former ion represents a stable fragment ion formed by loss of two hydrogens during cleavage of the sugar residue (structure 6, Scheme 1). The same phenomenon has been

observed for sulfated sugar head groups from animal lipids where an analogous fragment is seen at *m/z* 257, 2 mass units below the intact anion of a sulfated hexose (18). A small fragment at *m/z* 241 is also seen in the negative FAB spectrum of these 6-*O*-sulfated hexosyl lipids, but the ion at *m/z* 257 is twenty times more abundant. In addition, these compounds can be distinguished from SQD by the dominant fragment found in their spectra at *m/z* 97, $[HSO_4]^-$ (19). B/E linked scans on the precursor at *m/z* 241 from the spinach and the bacterial SQD samples showed a comparable pattern. Since parent ion selection is not precise with B/E scans on a double focusing instrument, the daughter ion at *m/z* 225 was proposed to be the dehydrated product of the minor sulfoquinovosyl ion at *m/z* 243, while the ion at *m/z* 80 corresponded to the SO_3^- radical anion (Scheme 1). The most abundant fragment at *m/z* 161 resulted from the loss of a neutral SO_3 group. Based on the limited available information, it was not possible to propose a precise structure for the latter fragment.

To complete the series of daughter ion scans and to confirm the identity of the proposed fragments, FAB-CAD-MS/MS scans for the ions *m/z* 225 and *m/z* 80 from the spinach sample were obtained. The fragment at *m/z* 225, as part of the sulfoquinovosyl head group, produced the SO_3^- fragment at *m/z* 80. In addition, several other fragments, including that at *m/z* 165, were generated. The ion at *m/z* 80 gave rise to the SO_2 ion with a mass of 64, confirming that this ion represents the sulfonate group.

CONCLUSION

The results indicate that FAB-MS is a useful technique for characterizing the composition of molecular species of sulfoquinovosyl diacylglycerol lipids. Prominent molecular ions are produced and key high mass fragments resulting from loss of the ketene analog of the *sn-2* acyl group provide information about the composition. Significant fragments are also present in the low mass end of the spectra which are characteristic of sulfoquinovosyl diacylglycerol. FAB-CAD-MS/MS can be used to confirm the identity of individual molecular species and to elucidate the fragmentation pathway of these compounds.

Based on the results obtained in this study, several diagnostic fragments from the negative FAB-MS of sulfolipid are proposed: first, the sulfoquinovose head group specific ions with masses of 225, 7, and 241/243, 6, and second, the sulfoquinovose glycerol specific deacylation products at *m/z* 283 and 299. The latter ion is sometimes weak in the B/E spectrum of the $[M-H]^-$ or $[M-H-R=C=O]^-$ ions. Since the daughter ion at *m/z* 299 did not give rise to the ion at *m/z* 241, and since the B/E linked scans using these two ions as precursors revealed distinct fragmentation patterns, it was postulated that both ions were produced in competing reactions from the molecular ion or monodeacylation products.

Comparison of linked B/E scans of the high mass ions and, in particular, of the head group specific ion at *m/z* 241, revealed similar fragmentation patterns for the spinach and bacterial sulfolipid, strongly suggesting that the sulfolipid found in both organisms is sulfoquinovosyl diacylglycerol. The data supported and extended earlier findings obtained by Wood *et al.* (4) and Radunz (6), who identified the sulfolipid in *R. sphaeroides* by co-chromatography with standards. However, it should be pointed out that the mass spectrometric technique used does not distinguish between the sulfoquinovose sugar and its stereoisomers. Future work in our laboratory with these compounds will focus on characterizing the positions of acyl group unsaturation from the high mass fragments in the FAB-CAD-MS/MS spectra.

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Positional Analyses of Triacylglycerol Fatty Acids in the Milk Fat of the Antarctic Fur Seal (*Arctocephalus gazella*)

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The positional distribution of fatty acids has been determined for the milk triacylglycerols of the Antarctic fur seal, *Arctocephalus gazella*. Of particular interest was the positional distribution of the polyunsaturated n-3 fatty acids in milk triacylglycerols (TG). In adipocytes of pinnipeds, TG are synthesized with the n-3 fatty acids primarily in the *sn*-1,3 positions. To determine the positional distribution, extracts of enzymatically digested lipids were separated by thin-layer chromatography, and the constituent fatty acids were separated and quantified by gas-liquid chromatography. Monoenoic and saturated fatty acids comprised over 75% of the total, the ratio of monoenoic to saturated fatty acids being 2:1. The percent content of the long-chain n-3 fatty acids, 20:5, 22:5 and 22:6, ranged between 15-20%. The positional analyses revealed that at the *sn*-2 position of milk TG, saturated fatty acids were in excess (57%), and the content of n-3 fatty acids was less than 5%. More than 80% of the n-3 fatty acids in milk were located in the *sn*-1,3 positions. The data indicate that in pinnipeds TG are synthesized in the mammary gland and adipose tissue with fatty acids having similar positional distributions.
Lipids 27, 637-639 (1992).

As a group, pinnipeds produce milk with an exceptionally high fat content (1-10), ranging from 19% in the Galapagos sea lion (1) to 61%, the highest value reported for any mammal, in the hooded seal (2). In the milk of the various species, 18:1 (17-47%), 16:0 (9-22%) and 16:1 (6-19%) (6-8,11-17) consistently were found to be the predominant three fatty acids. The polyenoic n-3 (20:5, 22:5 and 22:6) and the monoenoic (20:1 and 22:1) fatty acids common to the marine food web comprised between 15-40% of the total fatty acids in the milk of pinnipeds (6-8,11-13,16,17). Rather than being synthesized *de novo*, the polyenoic and most likely the long-chain monoenoic fatty acids are derived from the diet of the lactating female.

Brockerhoff and co-workers (18-20) showed that the fatty acid positional distribution in stored triacylglycerols (TG) were different in pinnipeds than in cold blooded organisms. Therefore, we were interested in determining whether the mammary gland of pinnipeds adaptively arranged the fatty acids in the milk TG to mimick the positional distribution in the stored fat of prey animals. To answer this question and to gain a better understanding of the utilization and processing of lipids in these animals, the positional distribution was determined for the fatty acids in the milk of the Antarctic fur seal (*Artocephalus gazella*). Our data on the milk TG indicate that there is no such adaptation. Overall, the positional distribution of the fatty acids is very similar to data reported previously for seal blubber (18-20).

METHODS

Milk samples (30-120 mL) were collected on Bird Island, South Georgia (54, 00'S, 38, 02'W) from four fur seals marked on the date of parturition. At the time of collection, the animals, which could be approached to within 1-2 m, were captured with a rope noose attached to a 3-m pole. Captured animals were then restrained using a specially constructed board as described by Gentry and Holt (21). Once restrained, females were weighed and then injected with 40 units of Oxytocin. After a few minutes, milk was manually expressed from the teat into plastic vials. Samples were kept frozen at −20°C until lipid analyses were carried out.

The milk lipids were extracted with chloroform/methanol (2:1, vol/vol). Extracts were weighed to obtain the percent lipid in the milk and then analyzed by thin-layer chromatography (TLC). The total fatty acids in the lipid extracts were methylated with boron trifluoride after undergoing saponification (22). The distribution of fatty acid methyl esters was determined by gas-liquid chromatography. Methyl esters were separated on a Durabond 225 column (0.00025 × 30 m, J&W Scientific, Folsom, CA) in a Hewlett-Packard (Kennet Square, PA) model 5890a equipped with a hydrogen flame detector. Column temperature was 220°C. The carrier gas was helium and the flow rate was 1.42 mL/min. The detector and injection port temperatures were 350°C. The resulting peak areas were measured electronically with a Hewlett-Packard 3392a and normalized to give values as weight percent of total fatty acid.

To determine the positional fatty acid distribution, the lipid extracts were first hydrolyzed with porcine pancreatic lipase (steapsin) in a 1.0 M Tris buffer (pH 8.0) as described by Mattson and Volpenhein (23). Following extraction, the enzymatic digestion products, 2-monoacylglycerols and free fatty acid released from the 1- and 3-positions, were separated by TLC. Using fatty acid and monoglycerol standards, both the digestion products and completion of the reaction could be monitored. The monoglycerols and free fatty acids were scraped from the plate and recovered after a subsequent TLC separation with only the standards being visualized with iodine vapor. Methylation of fatty acids and analyses of the resulting methyl esters for each of the recovered fractions were done as described above.

RESULTS

Analyses by TLC confirmed that the extracted lipid from fur seal milk consisted almost exclusively of TG. Although milk samples were obtained at different stages of the lactation cycle and the overall fat content in the milk varied from 30-52% (Table 1), individual variations in the percent content of the major fatty acids were quite small

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Abbreviations: TG, triacylglycerol; TLC, thin-layer chromatography.

TABLE 1

Percent Fat Content in the Milk of Four Antarctic Fur Seals

Female (no.)	Days postpartum	Percent fat
247	0	52.3%
263	0	42.6%
257	17	29.7%
235	32	39.5%

TABLE 2

A Comparison of the Fatty Acid Distributions in the Milk Triacylglycerols (TG)[a] of Four Antarctic Fur Seals and in the TG of Their Major Prey, *E. superba*

Fatty acid	Antarctic fur seals (no.)				*E. superba*[b] (weight percent)
	247	263	257	235	
	Weight percent				
14:0	3.7	4.7	6.4	6.1	14.6
16:0	18.4	18.6	18.4	17.9	23.0
16:1	9.3	9.8	10.6	10.2	9.0
17:1	1.0	1.0	1.1	1.0	0.5
18:0	1.7	1.8	2.0	2.0	1.1
18:1	36.0	32.2	36.6	35.5	20.0
18:2	1.7	1.7	1.7	1.5	0.2
18:3	0.5	0.5	0.7	0.4	0.3
20:1	4.9	4.1	4.5	2.7	0.8
20:4	0.7	0.7	0.4	0.5	0.5
20:5	7.1	8.7	6.9	12.1	13.7
22:1	1.3	1.3	0.9	0.6	—
22:5	2.0	2.1	2.2	2.4	0.2
22:6	7.1	8.0	6.0	5.5	8.0
Total	95.4	95.2	98.4	98.4	91.9

[a] Fatty acid distributions were obtained from a single analysis of each individual milk sample.

[b] Reference 24.

TABLE 3

Positional Distribution of Fatty Acids in Fur Seal Milk[a]

	sn-2 Position	*sn*-1,3 Positions
Saturated fatty acids		
14:0	11.4 (±4.3)	3.6 (±1.5)
16:0	44.9 (±1.8)	8.4 (±0.5)
18:0	0.5 (±0.05)	2.6 (±0.2)
Monoenoic fatty acids		
16:1	13.8 (±0.9)	9.2 (±1.2)
18:1	17.5 (±4.0)	41.5 (±3.9)
20:1	0.2 (±0.21)	5.7 (±1.0)
22:1		1.5 (±0.4)
Polyenoic fatty acids Group 2[b]		
18:2	2.2 (±0.3)	1.4 (±0.08)
18:3	0.6 (±0.17)	0.7 (±0.08)
20:4	0.3 (±0.19)	0.5 (±0.15)
Polyenoic fatty acids Group 2[c]		
20:5	2.2 (±0.4)	9.7 (±2.5)
22:5	0.2 (±0.26)	2.8 (±0.2)
22:6	1.6 (±0.8)	7.5 (±1.3)
Totals	95.4	95.1

[a] Percent content values are means obtained from the analyses of four individual samples of fur seal milk. Standard deviations are given in parentheses.

[b] Polyenoic fatty acids Group 1 consists of polyunsaturated fatty acids commonly found in the diet of terrestrial animals.

[c] Polyenoic fatty acids Group 2 consists of polyunsaturated fatty acids commonly found in the diet of marine animals.

(values are listed in Table 2). The fatty acids with less than 20 carbons comprised over 70% of the total. The remaining portion consisted primarily of 20:5 and 22:6, with the ratio of 20:5 to 22:6 varying from 1:2.2. The percentage of any one of the polyenoic fatty acids in Group 1 never exceeded 1.7%.

When the distribution at the *sn*-2 position was examined, a definite pattern could be discerned. Over 85% of these fatty acids were in order of relative amounts 16:0, 18:1, 16:1 and 14:0. From the data in Table 3, it can be calculated that 73% of 16:0 and 61% of 14:0 in milk TG were located at the *sn*-2 position. The corresponding values for 16:1 and 18:1 were 43% and 17%, respectively. A random arrangement would correspond to a value of 33% at the *sn*-2 position. In contrast, the percent content at the *sn*-2 position for any of the polyenoic fatty acids of Group 2 was low. At the *sn*-1,3 positions, 20% of the fatty acids in the TG of fur seal milk were 20:5, 22:5 and 22:6.

DISCUSSION

The high fat content in pinniped milk is related to their amphibious existence, which separates lactation on shore from feeding at sea (9,10). In the family *Otariidae*, consisting of fur seals and sea lions, lactation on shore is interspersed between feeding trips to sea. These periods of foraging typically last between 1 and 7 d and then are followed by a suckling period of 1 to 3 d on shore (10). In the case of the Antarctic fur seal, while at sea the female will derive 52% of her calories from the fat stored in krill (25). Such a diet enables the seal to provide her pup with milk enriched in lipids (26).

Our current study is the first to report on the partial positional distribution of the n-3 fatty acid in the milk of a pinniped. The polyenoic n-3, as well as the monoenoic C_{20} and C_{22} fatty acids, also common to the marine food web, were located primarily in the *sn*-1,3 positions. The *sn*-2 position contained a high percentage of 16:0 (45%). Overall, the distribution was essentially the same as reported previously for the TG of seal blubber (18–20). In seals, the *sn*-2 position was found to contain mostly saturated fatty acids, 14:0 and 16:0, with lesser amounts of monoenoic acids, 16:1 and 18:1. The n-3 fatty acids comprised only a small percentage of the fatty acids at this position and were located primarily at the *sn*-1,3 positions. On the other hand, in fish and invertebrate TG, the n-3 fatty acids were located primarily at the *sn*-2 position, and 14:0 and 16:0 were the two major fatty acids at the *sn*-1,3 positions (18,19). There was no adaptation in the mammary gland to provide suckling pups with dietary fats with positional distributions mimicking those found in future prey animals in the ocean.

Prior to our study, the fatty acids in the milk TG had been reported for three other species of otariids or

eared seals, the Northern fur seal *(Callorhinus ursinus)* (8), the California sea lion *(Zalophus californianus)* (16) and the Galapagos fur seal *(Arctocephalus galapagoensis)* (17). Many more studies have been done on phocids or earless seals (3,6,7,11-16). Our data on the total fatty acid composition are in general agreement with these other studies which show the milk fat to be enriched, particularly in monoenoic and saturated fatty acids (7,8,11-17). In most studies, including our own, the ratio of monoenoic to saturated fatty acid was approximately two. Exceptions to this trend were data obtained on the milk of the harp seal *(Pagophilus groenlandicus)*, hooded seal *(Cystophora cristata)* and the California sea lion. In the seal studies (3,6), the milk had a low content of saturated fatty acids resulting in the ratio having a value greater than 3.8. However, values reported by Cook and Baker (13) for harp seal milk agree with data obtained in other species. In the study of the California sea lion milk, the relative amounts of saturated and monoenoic acids were almost equal and the resulting ratio was slightly greater than one (16). Observed differences in the fatty acid composition of milk TG may be related to variations in the composition of the dietary fat of these animals. The long chain polyenoic acids are at comparable levels in both the milk and the principal prey, *Euphausia superba* (24), of the Antarctic fur seal (see Table 2).

In addition to obtaining data on the major energy source of suckling fur seals, our study may provide insights into ways to supplement infant formula diets with n-3 fatty acids. There is growing evidence that the n-3 fatty acids are essential in the neonatal period in humans (27,28). Studies have been done with formulas supplemented with fish oils as a source of 20:5 and 22:6 n-3 fatty acids (28). We currently are extending our studies of neonatal pinnipeds to determine whether variations in the location of n-3 fatty acids in dietary marine fats may be important. By studying animals before and after weaning, when they are switched to a diet of fish, we can determine the effects on both plasma membranes and lipoproteins, in particular on the size and number of chylomicra. Having natural diets with fats differing in the positional distribution of n-3 fatty acids, neonatal pinnipeds provide us with a unique animal model for addressing some of these issues.

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The Fatty Acids of the Sponge *Dysidea fragilis* from the Black Sea

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The fatty acid composition of the sponge, *Dysidea fragilis*, from the Black Sea has been determined by analytical gas chromatography, silver ion high-performance liquid chromatography and gas chromatography/mass spectrometry. More than a hundred different fatty acids were identified, of which many were similar to those in sponges from tropical seas. On the other hand, some of the fatty acids identified have not been found previously in sponges or other marine sources and perhaps are new to science. These include 13-methyl-tetradec-4-enoic and 14-methyl-hexadec-6-enoic acids, together with demospongic acids, *i.e.* 5,9,17-tetracosatrienoic, 5,9,17-pentacosatrienoic and 5,9,19-pentacosatrienoic acids. From the elution behavior on silver nitrate chromatography, all the double bonds were of the *cis*-configuration.
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Sponges are relatively primitive multicellular organisms which are known to contain a unique variety of distinctive fatty acids in the membrane phospholipids in unusual molecular species (reviewed by Djerassi and Lam (1)). In particular, these include the demospongic acids, *i.e.*, C_{24} to C_{30} fatty acids with 5,9-diunsaturation and often a further double bond in the terminal part of the molecule. Most of the published work has dealt with species from the Caribbean Sea or the Pacific Ocean. The Black Sea is an inland sea with reduced salinity (approximately half) compared to the oceans or the Mediterranean Sea; it also has a high concentration of hydrogen sulfide, reaching near saturation levels at depths below 200 m.

In this study, total fatty acids of a sponge, *Dysidea fragilis* (Family, Dysideidea; Order, Dictyoceratida), from the Black Sea have been examined by methods involving fractionation of the complex mixtures by degree of unsaturation by high-performance liquid chromatography (HPLC) in the silver ion mode, followed by gas chromatography/mass spectrometry (GC/MS) of the picolinyl ester derivatives. The methodology has been improved somewhat from an earlier study of the fatty acids of certain Black Sea invertebrates (2).

MATERIALS AND METHODS

Sample. The sponge *Dysidea fragilis* was collected from the Black Sea, at depths of 2 to 10 m, near the town of Pomorie in Bulgaria in August 1989. Samples were washed in tap water and immediately immersed in ethanol for transport to the laboratory. Lipids were extracted with chloroform/methanol (2:1, vol/vol) and saponified, the non-saponifiables were removed, and the acids were methylated with methanol containing 1% sulfuric acid (3). The yield of lipid was 0.36% of the wet weight of the sponge (from 10 sponges, total weight 160 g). Voucher specimens were deposited in the Museum of Natural History, Sofia, Bulgaria by Dr. St. Andreev.

Analytical gas chromatography. For analytical purposes, a Carlo Erba Model 4130 capillary gas chromatograph (Erba Science, Swindon, U.K.), fitted with split/splitless injection, and equipped with a capillary column (0.25 mm i.d. × 25 m in. length) of fused silica coated with Carbowax 20M (Chrompack UK Ltd., London, U.K.), was used with the methyl ester derivatives. After holding the temperature at 170°C for 3 min, it was temperature-programmed at 3°C/min to 225°C, then was held at this point for a further 17 min. Hydrogen was the carrier gas. Components were quantified by electronic integration. Equivalent chain length (ECL) values were determined isothermally at 170°C (C_{13} to C_{22} components) or at 200°C (C_{23} to C_{27} components).

Silver ion high-performance liquid chromatography. A Spectra-Physics Model 8700 solvent delivery system was used (Spectra-Physics Ltd., St. Albans, U.K.), together with a Cunow model DDL 21 detector (Severn Analytical Ltd., Shefford, Beds, U.K.). A stream-splitter (approx. 10:1) was inserted between the column and the detector to enable collection of fractions. The silver ion column, Chrom-Spher Lipids™, was kindly donated by Dr. Stephan Rose of Chrompack Ltd. (Middelburg, Netherlands) and was prepared as described elsewhere (4). For micro-preparative purposes, 1–2 mg of methyl esters were applied to the column in 10 μL of dichloroethane. The column was eluted using a binary gradient procedure with mixtures of dichloromethane/dichloroethane (1:1, vol/vol) (Solvent A) and dichloromethane/dichloroethane/methanol/acetonitrile (45:45:5:5, by vol) (Solvent B). There was a linear gradient from 100% A to 95% A/5% B over 15 min then to 80% A/20% B over a further 25 min and finally to 50% A/50% B over another 10 min at a flow-rate of 1 mL/min.

Gas chromatography/mass spectrometry. The fatty acids were subjected to GC/MS as the picolinyl esters prepared essentially by the method of Balazy and Nies (5). A Hewlett-Packard Model 5890 gas chromatograph attached to a Hewlett-Packard Model 5970 Mass Selective Detector (Hewlett-Packard Ltd., Wokingham, U.K.) was used. The picolinyl esters were analyzed on a fused-silica capillary column (25 m × 0.25 mm i.d.), coated with a cross-linked 5% phenylmethylsilicone (CP-Sil 8™, Chrompack UK Ltd., London, U.K.), and injected onto the column at 70°C (held for 0.5 min), temperature-programmed to 210°C at 60°C/min, held there for 3 min, raised again to 280°C at 2°C/min, then held for a further 40 min. The column outlet was connected directly to the ion source of the mass spectrometer, which was operated at an ionization energy of 70 eV.

RESULTS

In the present study, the total fatty acids from the sponge *Dysidea fragilis* were analyzed, as opposed to those of the phospholipid fraction, which has a high proportion of choline and ethanolamine plasmalogens (Stef-

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Abbeviations: ECL, equivalent chain length; GC, gas chromatography; HPLC, high-performance liquid chromatograhy; MS, mass spectrometry.

anov, K., unpublished). As anticipated, the GC trace of the methyl ester derivatives of the fatty acids was highly complex, with well over 100 distinct peaks being resolved, many with much longer retention times than are normally seen in fatty acid methyl esters derived from mammalian tissues. GC/MS of picolinyl esters has been proven of great value for the determination of fatty acid structures in complex samples (for reviews, see refs. 3,6), but some loss of resolution is inevitable when they are subjected to GC separation; their high polarity and molecular weight make it advisable to use non-polar stationary phases, especially when the samples contain fatty acids with a higher molecular weight than normal. Therefore, the methyl ester derivatives were fractionated by silver ion HPLC for examination by analytical GC, and for conversion to the picolinyl esters for GC/MS. This approach has assisted greatly in the elucidation of many complex samples of natural origin (2,7–9). The chromatographic trace from the silver ion HPLC is shown in Figure 1, and the points where fractions were collected are indicated. Resolution was achieved mainly according to the number of double bonds in each component, and partly by their configuration and position. Ten fractions were collected for derivatization and analysis by GC/MS. Those fatty acids identified, and their relative proportions and equivalent chain lengths (28) are given in Table 1 together with references to previous reports of their occurrence in sponges and marine invertebrates. Ninety different fatty acids were identified and are listed if they occur at a concentration of 0.05% or greater, and a further 13 fatty acids were detected in even smaller proportions.

The saturated fatty acids (fraction 1) were mainly 14:0, 16:0 and 18:0 with small amounts of other straight-chain fatty acids and *iso*- and *anteiso*-methyl branched acids. In addition, there was a small amount of 4,8,12-trimethyl-13:0, which is common in marine invertebrates, phytanic acid, other monomethyl-branched components, and a cyclopropane fatty acid, some of which do not appear to have been reported to occur in sponges.

The second minor fraction contained monoenoic acids, which probably have double bonds of the *trans*-configuration (insufficient material was available for confirmation), since they eluted well ahead of the conventional *cis*-monoenes. The 6-16:1 and 9-16:1 fatty acids were identified.

cis-Monoenes were in the third fraction and the main components were conventional positional isomers of 16:1, 18:1, 20:1 and 22:1, together with related odd-chain components. There were also high proportions of C_{24} to C_{26} monoenes, some of which do not appear to have been reported previously for sponges. Small amounts of monoenes with double bonds in position 5, that may be related biosynthetically to the dienes and trienes described below, were found. In addition, monoenoic fatty acids with methyl branching were identified, some of which appear to be novel. The most abundant of these was 15-methyl-hexadec-6-enoic acid, which has been reported previously to occur in human *Vernix caseosa* (29) and which was recently confirmed in human cerumen by mass spectrometry of its picolinyl ester (30), but has not been found before in marine invertebrates. As an example, Figure 2 shows the mass spectrum of the picolinyl ester derivative of 14-methylhexadec-6-enoic acid, which has not been found before in marine samples. The molecular ion

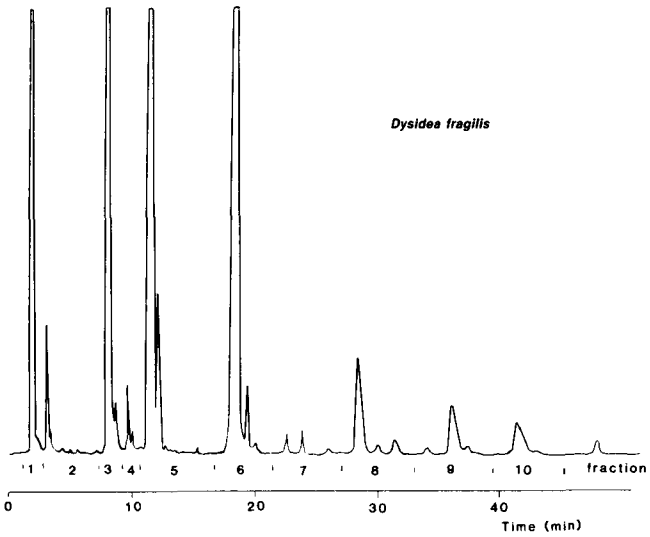

FIG. 1. Silver ion HPLC of methyl esters of the fatty acids from *Dysidea fragilis*. See Materials and Methods for experimental details. Numbered fractions were collected between the points indicated.

(m/z = 359) is that expected of a C_{17} monoenoic acid (although the GC retention time was appreciably less than that of most 17:1 isomers). The gap of 28 amu between m/z = 302 and 330 is indicative of an *anteiso*-methyl branch (31), while the abundant ion at m/z = 246 is a characteristic marker for a double bond in position 6 (32). Isomeric compounds, in which either the position of the methyl branch or of the double bond varied, and homologues of this acid were also found, *i.e.*, 13-methyl-4-14:1 and 15-methyl-9-16:1. The former had a molecular ion at m/z = 331, a gap of 28 amu between m/z = 288 and 316 for the iso-methyl branch; the double bond in position 4 was indicted by a prominent ion at m/z = 218 while the base peak was at m/z = 151 (32). Only the 15-methyl-9-16:1 fatty acid appears to have been reported previously to occur in marine sources (12,24).

The minor fraction 4 contained mainly non-methylene-interrupted dienes, many but not all of which were similar to those in the following fraction, a finding that suggests that one of the double bonds might be of the *trans* configuration. Insufficient material was available to confirm this hypothesis.

Fraction 5 contained most of the dienoic components, including conventional methylene-interrupted fatty acids of the n-4 and n-6 families, such as 18:2n-6, and dienes with more than one methylene group between the double bonds and up to 27 carbon atoms in the chain ("demospongic acids"). With most of the latter group, the first double bond was in position 5 and there were two central methylene groups, *i.e.*, the most common isomers were 5,9-dienoic acids. The mass spectrum of the picolinyl ester derivative of 5,9-hexacosadienoic acid is illustrated in Figure 3A, as an example. As usual, there was an abundant molecular ion (m/z = 483). The most important diagnostic feature was an ion formed by cleavage between the two methylene groups separating the double bonds which, being very unusual, for a picolinyl ester, has an odd mass number, *i.e.* in this instance at m/z = 219 (33). A

TABLE 1

The Fatty Acid Composition (wt% of the total) from the Sponge *Dysidea fragilis*, Equivalent Chain Length (ECL) Values and Fraction Number, Together with References to Previous Reports of Their Occurrence in Sponges and Other Marine Invertebrates

Fatty acid[a]	Wt%	Fraction number	ECL	Previous references[b]
12-methyl-13:0	0.1	1	13.50	23
14:0	1.2	1	14.0	21
4,8,12-trimethyl-13:0	0.1	1	14.04	18, 22
7-14:1	0.1	3	14.72	14
13-methyl-4-14:1	0.6	3	14.75	
i-14:0	1.5	1	14.51	20, 24
ai-14:0	0.5	1	14.65	19, 23
15:0	0.4	1	15.0	11
i-15:0	1.7	1	15.52	22, 24
ai-15:0	0.4	1	15.67	22, 24
16:0	7.7	1	16.0	11
5-16:1	0.1	3	16.10	25
7-16:1	0.6	3	16.18	10
9-16:1	2.4	3	16.25	10
11-16:1	0.1	3	16.36	12
15-methyl-6-16:1	0.2	3	16.62	
15-methyl-9-16:1	0.1	3	16.77	12, 24
14-methyl-6-16:1	0.1	3	16.82	
10-methyl-16:0	0.1	1	16.2	12
i-16:0	2.2	1	16.53	19, 24
ai-16:0	1.7	1	16.67	19, 24
7,10-16:2	0.1	5	16.68	10
9,12-16:2	0.1	5	16.68	10
9,10-methylene-16:0	0.3	1	16.79	14
6,9,12-16:3	0.2	7	16.99	10
7,10,13-16:3	0.2	7	17.09	10
17:0	0.5	1	17.0	11
9-17:1	0.1	3	17.25	12, 25
11-17:1	0.2	3	17.36	12
14-methyl-17:0	0.2	1	17.48	2
16-methyl-17:0	0.1	1	17.53	
15-methyl-17:0	0.1	1	17.67	
phytanic acid	0.2	1	17.04	19
18:0	1.5	1	18.0	11
5-18:1	0.1	3	18:09	2, 11
9-18:1	3.1	3	18.16	10
11-18:1	7.1	3	18.23	10
15-methyl-18:0	0.5	1	18.48	
16-methyl-18:0	0.3	1	18.54	13, 19
17-methyl-18:0	0.1	1	18.68	13, 18
11,12-methylene-18:0	0.4	1	18.8	14
5,8-18:2	0.1	5	18.4	
9,12-18:2	2.0	5	18.57	10
11,14-18:2	0.2	5	18.69	2, 10
6,9,12-18:3	0.5	7	18.84	2, 10
9,12,15-18:3	1.1	7	19.17	2, 10
6,9,12,15-18:4	0.9	8	19.45	10
19:0	0.1	1	19.0	11
16-methyl-19:0	0.1	1	19.49	
11-19:1	0.1	3	19.22	12
20:0	0.1	1	20.0	11
7-20:1	0.3	3	20.08	2
11-20:1	tr	3	20.15	10, 23
13-20:1	0.3	3	20.22	17
15-20:1	0.2	3	20.34	10
5,11-20:2	0.2	5	20.35	2, 16
5,13-20:2	tr	5	20.43	2, 16
11,14-20:2	tr	5	20.64	10
8,11,14-20:3	0.5	7	20.74	10
11,14,17-20:3	tr	7	20.96	10
5,8,11,14-20:4	3.8	8	20.92	10
8,11,14,17-20:4	0.5	8	21.33	10
5,8,11,14,17-20:5	2.9	9	21.51	10
6,9,12,15,18-21:5	0.1	9	22.50	2
22:0	0.6	1	22.0	11
5-22:1	0.1	3	22.11	
15-22:1	1.1	3	22.19	17, 23
7,15-22:2	0.3	5	22.44	10
7,10,13,16-22:4	0.5	8	22.86	11
4,7,10,13,16-22:5	0.7	9	23.25	10
7,10,13,16,19-22:5	0.8	9	23.60	10
4,7,10,13,16,-22:6	2.9	10	23.84	10
9-24:1	0.1	3	20.08	
17-24:1	4.4	3	24.26	17, 21
7,17-24:2	0.1	5	24.34	
5,9-24:2	0.2	5	24.36	21, 23
23-methyl-5,9-24:2	0.1	5	24.79	26
5,9,17-24:3	0.2	6	24.70	
9-25:1	0.1	3	25.07	
5,9-25:2	3.7	5	25.21	19, 21, 22
24-methyl-5,9-25:2	0.8	5	25.73	26, 27
5,9,17-25:3	0.1	6	25.70	
5,9,19-25:3	0.2	6	25.80	
9-26:1	0.4	3	25.10	15, 17
5,9-26:2	9.4	5	26.20	1, 19, 21
9,19-26:2	0.6	5	26.28	17
5,9,17-26:3	1.2	6	26.73	17
5,9,19-26:3	14.1	6	26.85	17
5,9-27:2	0.8	5	27.20	23

[a]The number of carbons listed does not include the methyl branch In addition, trace amounts of 13-methyl-16:0, 21:0, 22:0, 23:0, 24:0, *trans*-monoenes (6-16:1 and 9-16:1), *cis*-monoenes (13-methyl-14:1, 4-16:1, 7-17:1, 13-22:1 and 16-23:1) and 24:5n-3 were identified.

[b]For common fatty acids, such as 16:0, only a representative reference is given.

similar feature was reported for the spectrum of the picolinyl ester of 5,9,12-octadecatrienoic acid (34). In addition, gaps of 26 amu between *m/z* = 178 and 204, and between 232 and 258, confirmed the presence of double bonds in positions 5 and 9, respectively. The presence of a regular series of ions 14 amu apart, from *m/z* = 258 to 468, indicated that there were no further double bonds or any branch points in the chain. Fatty acids with the 5,9-dienoic structure have been found in many sponges from tropical seas (1). Related fatty acids were present with an *iso*-methyl branch, *i.e.*, 23-methyl-5,9-24:2 and 24-methyl-5,9-25:2, which have been found previously in sponges (26,17). In addition, several fatty acids were found in which there were more than two methylene groups between the double bonds. Interpretation of the mass spectrum of the picolinyl esters of these fatty acids is sometimes less straight-forward, but is relatively simple when comparison with model compounds is possible, as was here (2,33). Most, if not all of these compounds have previously been found in other sponges or marine invertebrates.

Fraction 6 from the silver ion column is a trienoic fraction in which the double bonds all have more than one methylene group separating them. The most abundant single component was 5,9,19-hexacosatrienoic acid, and the mass spectrum of the picolinyl ester is shown in Figure 3B. The molecular ion at *m/z* = 481 was prominent, and the ions diagnostic for the 5,9-double bonds were clearly seen, as in the previous example. However, a gap of 26 amu between *m/z* = 370 and 396 confirmed the presence of the double bond in position 19. Regular series of ions 14 amu apart between *m/z* = 258 and 370 and between *m/z* = 396 and 466 showed that no double bonds or branch points were present in other parts of the molecule. Again, this

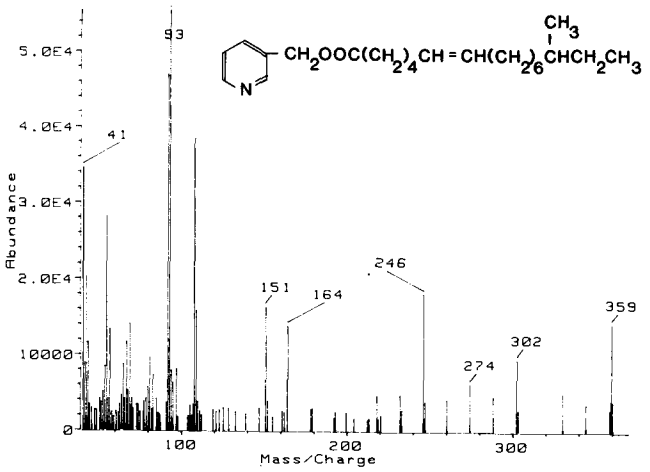

FIG. 2. Mass spectrum of the picolinyl ester of 14-methylhexadec-6-enoic acid.

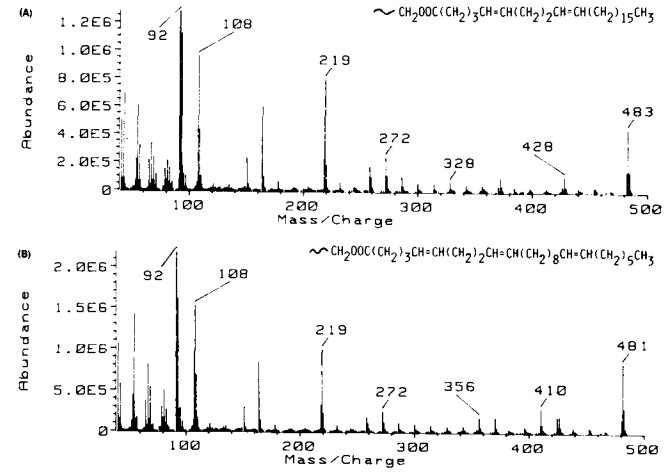

FIG. 3. Mass spectra of the picolinyl esters of 5,9-hexacosadienoic acid (A) and 5,9,19-hexacosatrienoic acid (B).

fatty acid has been found in sponges from tropical seas (1). However, certain of the other isomers and homologues, that were found albeit in small amounts, *i.e.* 5,9,17-24:3, 5,9,17-25:3, 5,9,19-25:3, do not appear to have been reported previously. It was not easy to obtain definitive mass spectra from components present in such small amounts, but identification was simple by comparison with the spectrum of the abundant homologue illustrated (Fig. 3B). Thus molecular ions at m/z = 453 and 467 were obtained for the 24:3 and 25:3 homologues, respectively, ions for the 5,9-double bond system or an isolated 19-double bond were present in the same positions, while the gap of 26 amu for the double bond in position 17 was between ions at m/z = 342 and 368.

Fraction 7 from the silver ion column contained three distinct peaks, from which six components emerged upon GC/MS of the picolinyl derivatives. These were found to be the common range of C_{16} to C_{20} trienoic acids with methylene-interrupted double bonds, *i.e.* mainly of the n-6 and n-3 families found in marine oils. Similarly, fraction 8 contained three peaks of which the first and most abundant was arachidonic acid, with 18:4n-3, 20:4n-3 and 22:4n-6 also identified. Fractions 9 and 10 then were found to contain the common range of penta- and hexaenoic acids, respectively.

DISCUSSION

In spite of its remoteness from species in the major oceans of the world, the sponge *Dysidea fragilis* contains many of the well-known demospongic acids found in the tropical seas around the United States of America (1) and apparently in Siberian and Arctic waters (35–39). The most abundant components found in this instance are common to many species, but it does appear to be unusual for so many different demospongic acids to be found in one species. Three trienoic acids, 5,9,17-24:3, 5,9,17-25:3 and 5,9,19-25:3, do not appear to have been recorded previously. From the chromatographic behavior on silver ion HPLC and by analogy with other such acids, the double bonds are presumed to be of the *cis*-configuration. Carballeira *et al.* (18) have stated that "it is very rare to find three consecutive acids with the 5,9-unsaturation together in the same sponge" (18). In this species, four such dienoic (24:2, 25:2, 26:2 and 27:2) together with two branched isomers and three consecutive trienoic acids (plus two isomers) were detected.

It has also been suggested (18) that sponges may be selective as to which of the isoprenoid fatty acids, 4,8,12-trimethyltridecanoic and 3,7,11,15-tetramethylhexadecanoic (phytanic), were incorporated into their tissues. In *Dysidea fragilis*, both of these were found.

A number of long-chain monoenoic fatty acids with double bonds in positions 5, 7 and 9 were found, that do not appear to have been recorded previously, but they can be presumed to have biosynthetic relationships to the demospongic acids. In addition, several branched-chain monoenoic acids with *cis*-double bonds in positions 4 and 6, *i.e.* 13-methyl-4-14:1, 14-methyl-6-16:1 and 15-methyl-6-16:1, of which the first two may be new to science, were detected. These do not have any obvious biosynthetic relationship to the other fatty acids found, and they were not detected in comprehensive studies of other Black Sea organisms (2,7).

Some adventitious contamination with marine algae which adhere to the sponge can occur, so there is a possibility that some of the fatty acids in the sample could have come from this source. However, no sponges which were obviously contaminated were collected. Algae also serve as a food for sponges so their fatty acids may be incorporated into sponge lipids by this route, though an examination of the composition of a number of marine algae from the Black Sea (7) showed mainly the polyunsaturated fatty acids of the n-3 and n-6 families. Some of the branched-chain, and especially the cyclopropane fatty acids, may have come from bacteria in the food chain or as symbionts (40,41).

The methodology utilized in this study is extremely powerful, and gave detailed structural information permitting complete structure elucidations of more than a hundred different fatty acids in this single species. Virtually every component present at a concentration of more than 0.05% was identified. Some of these do not appear to have been found previously in nature. Mass spectro-

metry of picolinyl esters (6) enables identification of fatty acids with much greater ease than has been possible with pyrolidides which have been used hitherto, especially with sponges. In addition, preliminary fractionation by silver ion HPLC affords clean fractions that greatly improves the resolution attainable on GC/MS.

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Dr. S. Andreev of the Museum of Natural History of the Bulgarian Academy of Sciences, Sofia, Bulgaria collected and classified the sponge. This research was funded in part by The Scottish Office Agriculture and Fisheries Department and by Project 344 with the Ministry of Science and Higher Education of Bulgaria.

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Preparation, Chromatography and Mass Spectrometry of Cholesteryl Ester and Glycerolipid-Bound Aldehydes[1]

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To identify lipid ester core aldehydes (aldehydes still bound to parent lipid molecules) among the secondary products of peroxidation, we have prepared reference standards of cholesteryl esters, diacylglycerols and the common glycerophospholipids (GPL) containing an aldehyde function. We subjected the unsaturated lipid esters to hydroxylation with osmium tetroxide and to carbon-carbon bond cleavage with periodic acid. The resulting aldehyde esters (mainly 5-oxovalerates and 9-oxononanoates of cholesterol and the glycerolipids) were isolated and purified by thin-layer chromatography (TLC) and reverse-phase high-performance liquid chromatography (HPLC). Liquid chromatography/mass spectrometry (LC/MS) was used to identify the cholesteryl and other neutral lipid ester aldehydes as the dinitrophenylhydrazones (DNPH). The choline (CGPL) and ethanolamine (EGPL) glycerophospholipids containing core aldehydes were identified by fast atom bombardment mass spectrometry (FAB-MS) and by gas chromatography/mass spectrometry (GC/MS) of the aldehyde-containing diacylglycerol moieties following dephosphorylation with phospholipase C and preparation of the methoximes (MOX) and the trimethylsilyl (TMS) ethers. The lipid esters containing the C_5 and C_9 aldehydes are chemically stable compounds with excellent chromatographic properties yielding mass spectra characteristic of the parent compounds. The overall yield of the core aldehydes was 10–20% of the unsaturated starting material.
Lipids 27, 645–650 (1992).

Recently much attention has been focused on the role of lipid peroxidation in many pathological conditions (2). Analysis of specific products of lipid peroxidation is crucial for the identification of pathological mediators in these instances. The initial step of lipid peroxidation is the conversion of polyunsaturated fatty acids into hydroperoxides. The hydroperoxides are then decomposed into aldehydes by homolytic scission of the two carbon bonds on either side of the hydroperoxy group (3). Because polyunsaturated fatty acids in biological systems are found in glycerophospholipids (GPL), triacylglycerols and cholesteryl esters, cleavage of the carbon-carbon bonds of the hydroperoxides would theoretically yield two types of aldehydes: a water-soluble aldehyde derived from the methyl terminal of the unsaturated fatty acid and a lipid soluble core aldehyde still containing most of the parent lipid molecule. The latter group of aldehydes, lipid ester core aldehydes, have rarely been studied as part of the peroxidation process (4). An exception is provided by the identification of 5-oxovaleroyl glycerophosphocholine (GPC) among the *in vitro* peroxidation products of 2-arachidonoyl GPC (5). In addition, GPC with azelaic acid in the *sn*-2 position has been isolated as one of the cytotoxic products generated during oxyhemoglobin-induced peroxidation of unsaturated choline glycerophospholipids (CGPL) (6). However, the core aldehydes of diacylglycerols, triacylglycerols and CGPL have been obtained in the past by ozonolysis and reductive cleavage during structural investigations of natural glycerolipids (7). In order to facilitate the studies on the core aldehydes in peroxidation mixtures, we have prepared model compounds from unsaturated cholesteryl esters, diacylglycerols and the common GPL, and have investigated methods for their rapid isolation and identification.

MATERIALS AND METHODS

Materials. The palmitic, oleic and arachidonic acid esters of cholesterol, 1-palmitoyl-2-oleoyl glycerophosphoethanolamine (GPE), egg yolk phosphatidylcholine (PC), phospholipase C *(Clostridium welchii),* and periodic acid were from Sigma Chemical Co. (St. Louis, MO). Methoxylamine HCl (MOX reagent), trimethylsilylchloride (TMSC), *N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and 1-fluoro-2,4-dinitrobenzene were from Pierce Chemical Co. (Rockford, IL). Osmium tetroxide was obtained from British Drug Houses, Ltd. (Toronto, Canada), while 2,4-dinitrophenylhydrazine was from Aldrich Chemical Co. (Milwaukee, WI). Propionitrile was from Romil (Loughborough, England). Other solvents and chemicals were of reagent grade or better quality and were obtained from local suppliers.

Preparation of cholesteryl oxoalkanoates. Cholesteryl 5-oxovalerate and cholesteryl 9-oxononanoate were prepared from cholesteryl arachidonate and cholesteryl oleate, respectively. To a solution of 1.5 mg of the unsaturated cholesteryl ester in 0.2 mL of pyridine/dioxane (1:8, vol/vol), 0.2 mL of a dioxane solution of osmium tetroxide (10 mg/mL) was added. After 2 h at room temperature, 6 mL of a suspension of sodium sulfite (made by adding 8.5 mL of 16% sodium sulfite to 2.5 mL of methanol) was added and mixed (8). After 1.5 h at room temperature, the mixture was centrifuged and lipids were extracted from the residue with chloroform/methanol (2:1, vol/vol). The lipid extracts containing the alcohols were evaporated under nitrogen and dissolved in 0.5 mL 95% ethanol. To this solution, 0.5 mL of 1% periodic acid in 95% ethanol was added, mixed and left at room temperature for 1 h

[1]Part of this work was presented at the Annual AOCS Meeting, Chicago, IL, May, 1991 (see ref. 1).

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Abbreviations: BSTFA, (trimethylsilyl)trifluoroacetamide; CI, chemical ionization; CGPL, choline glycerophospholipid; DNP, dinitrophenyl; DNPH, dinitrophenylhydrazone; EGPL, ethanolamine glycerophospholipid; EI, electron impact ionization; FAB-MS, fast atom bombardment mass spectrometry; GLC, gas-liquid chromatography; GC/MS, gas chromatography/mass spectrometry; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPL, glycerophospholipid; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; MOX, methoxime; NCI, negative chemical ionization; PC, phosphatidylcholine; PE, phosphatidyletanolamine; TLC, thin-layer chromatography; TMS, trimethylsilyl; UV, ultraviolet.

(9). The mixtures were diluted with 2 mL of chloroform and washed three times with water. The lower chloroform phase containing the cholesteryl ester core aldehydes was evaporated under nitrogen and the aldehydes were purified by thin-layer chromatography (TLC) on silica gel H using heptane/diisopropyl ether/glacial acetic acid (60:40:4, vol/vol/vol) as the developing solvent. The lipids were localized by spraying the plate with 2',7'-dichlorofluorescein in methanol or with Schiff's reagent (10). The fluorescent silica gel zones were scraped off the plate and eluted with chloroform/methanol (2:1, vol/vol). The extracts were reduced to a small volume and the lipid fractions characterized (see below).

Preparation of oxoalkanoates of GPL. The 1-palmitoyl-2-(9-oxononanoyl)- and 1-stearoyl-2-(9-oxononanoyl)-GPC were prepared from egg yolk PC as described for the preparation of the cholesteryl ester core aldehydes. The core aldehydes derived from PC were purified by TLC on silica gel H using chloroform/methanol/water (65:35:6, vol/vol/vol) as the developing solvent. The fluorescent silica gel zones were scraped off the plate and the lipids were eluted with chloroform/methanol (2:1, vol/vol). The phospholipid extract was reduced to a small volume and hydrolyzed with phospholipase C for 1 h at 37°C as previously described (11). After solvent evaporation, the diacylglycerol core aldehydes were characterized by various chromatographic and mass spectrometric methods. The 1-palmitoyl-2-(9-oxononanoyl) GPE was prepared as described for the corresponding PC-derived core aldehyde, except that the PE was first converted to the dinitrophenyl (DNP) derivative by treatment with 1-fluoro-2,4-dinitrobenzene to facilitate detection of cleavage product in high-performance liquid chromatography (HPLC) (12). The core aldehyde was purified by TLC using chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol). The UV absorbing silica gel zones were scraped off and extracted with chloroform/methanol (2:1, vol/vol). After solvent evaporation, the core aldehyde of the DNP-GPE product was characterized before and after derivatization.

Preparation of methoxime (MOX) derivatives. The MOX derivatives were obtained by heating the core aldehydes with 100 μL of the methoxylamine·HCl reagent at 60°C for 3 h (13). The reaction mixture was evaporated under nitrogen and the residue extracted with 5 mL of hexane and washed with water. The solvent layer was dried over sodium sulfate and evaporated to a small volume before gas-liquid chromatography (GLC) and gas chromatography/mass spectrometry (GC/MS).

Preparation of dinitrophenylhydrazone (DNPH) derivatives. The lipid ester core aldehydes were converted into the 2,4-DNPH derivatives using a modification of the method of Esterbauer and Chesseman (14). The aldehydes were treated with 0.5 mL of freshly prepared 2,4-dinitrophenylhydrazine in 1N HCl (0.5 mg/mL), mixed vigorously and kept in the dark for 2 h at room temperature and 1 h at 4°C. The reaction mixture was extracted with chloroform/methanol (2:1, vol/vol) and the recovered hydrazones were purified by TLC using a double development with dichloromethane (up to a height of 10 cm) and, after solvent evaporation, in the same direction with toluene (up to 17 cm). The ultraviolet (UV) absorbing silica gel zones were scraped off, extracted with methanol and reduced to a small volume. The DNPH derivatives of the CGPL core aldehydes recovered from the origin of the TLC plate were treated with phospholipase C for 2 h at 37°C, and the released DNPH derivatives of the diacylglycerol core aldehydes were purified by TLC using chloroform/methanol (95:5, vol/vol) as the developing solvent.

GLC and GC/MS. The core aldehydes of the cholesteryl esters and diacylglycerols, as well as their trimethylsilyl (TMS) ethers and MOX derivatives were resolved on basis of molecular weight by GLC on non-polar capillary columns as described by Myher and Kuksis (15). The TMS ethers were prepared by treating the lipid fractions or their MOX derivatives with a silylating reagent made up of one part BSTFA + 1% TMSC and one part pyridine for 30 min at room temperature. Nonpolar capillary GC/MS with electron impact (EI) or chemical ionization (CI) with ammonia was performed as previously reported (16).

HPLC and liquid chromatography/mass spectrometry (LC/MS). Reverse-phase HPLC with the DNPH derivatives of the core aldehydes was performed on a Supelcosil LC-18 column (250 mm × 4.6 mm) (Supelco, Inc., Bellefonte, PA) using acetonitrile/isopropanol (4:1, vol/vol) (1.0 mL/min) or a linear gradient of 30–90% propionitrile in acetonitrile (1.5 mL/min) as the eluting solvent (17). The peaks were monitored at 358 nm with a variable wavelength detector. Reverse-phase HPLC of the DNP-phosphatidylethanolamine (PE) core aldehydes was performed on a Supelcosil LC-18 column using 10 mM ammonium acetate, pH 5.0, and methanol as the mobile phase, beginning with a 5-min isocratic elution at 84% methanol, followed by a linear increase to 87% methanol within 15 min (12). Elution was monitored by measuring absorptivity at 348 nm and fractions were collected for fast atom bombardment-mass spectrometry (FAB-MS) examination.

FAB-MS. FAB-MS on the DNP-GPE core aldehydes and their diethyl acetals was performed using thioglycerol as the matrix as previously described (18).

RESULTS AND DISCUSSION

Characterization of cholesteryl ester core aldehydes. Figure 1 shows the TLC separation of the osmium tetroxide/periodic acid oxidation products of the palmitic, oleic and arachidonic acid esters of cholesterol. Lane A shows the osmium tetroxide oxidation products of cholesteryl palmitate. In addition to the band corresponding to the original cholesteryl ester (R_f 0.83), there is a more slowly migrating band representing the ester containing an oxidized sterol ring (R_f 0.26). Lane B contains the osmium tetroxide/periodic acid oxidation products of cholesteryl oleate. Separate bands are seen for the C_9 aldehyde (R_f 0.41) and its diethyl acetal (R_f 0.57), as well as the osmic acid ester (R_f 0.31) of cholesteryl dihydroxyoctadecanoate. Lane C contains the oxidation products of cholesteryl arachidonate. In this instance, the band corresponding to the original ester did not contain any residual cholesteryl arachidonate, the fluorescence being due to some other component of low mass. There were also bands near the origin of the TLC plate, which were subsequently shown to be due to the sterol ring oxidation products of the oxoester (Kamido, Kuksis, Marai and Myher, 1992, unpublished data). The bands with intermediate retention values were due to the C_5 aldehyde (R_f 0.28) and its diethyl acetal (R_f 0.34). There are other minor bands which have thus far remained unidentified. The band in

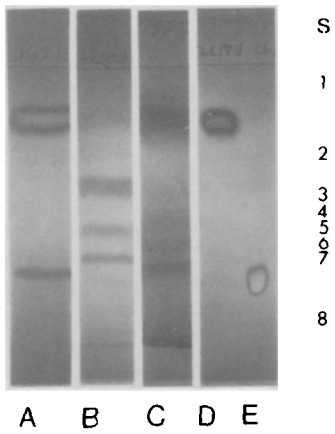

FIG. 1. TLC separation of osmium tetroxide/periodic acid oxidation products of palmitic (A), oleic (B) and arachidonic (C) acid esters of cholesterol. Lanes D and E contain cholesteryl oleate and free cholesterol, respectively. The lipid bands are identified as follows: 1, cholesteryl ester; 2, diethyl acetal of cholesteryl ester C_9 core aldehyde; 3, cholesteryl ester C_9 core aldehyde; 4, diethyl acetal of cholesteryl ester C_5 core aldehyde; 5, osmic acid ester of cholesterol dihydroxyoctadecanoate; 6, cholesteryl ester C_5 core aldehyde; 7, free cholesterol and oxo-cholesteryl palmitate; 8, origin and unidentified more polar oxidation products. Solvent system: heptane/diisopropyl ether/glacial acetic acid (60:40:4, vol/vol/vol). Lipid fractions were located by spraying with 2′,7′-dichlorofluorescein and Schiff's reagent (10).

Lane D is due to standard cholesteryl oleate (R_f 0.83) while that in Lane E is due to free cholesterol (R_f 0.26). The identity of the TLC fractions was established by chromatographic and mass spectrometric methods.

Figure 2 shows the nonpolar capillary GLC profiles of the C_5 (Fig. 2A) and C_9 (Fig. 2B) cholesteryl ester core aldehydes recovered from the TLC bands with R_f values 0.28 and 0.41, respectively, when analyzed as the free aldehydes. A single peak is seen for the C_9 oxidation product of cholesteryl oleate, while cholesteryl arachidonate gives, in addition to the major C_5 peak, one or two smaller peaks, which were not identified. A preparation of the MOX and TMS derivatives resulted in a uniform shifting of these peaks to slightly higher retention times, while a GC/MS examination of the peaks with ammonia CI gave pseudomolecular ions corresponding to $[M + NH_4]^+$ of the core aldehyde and its MOX derivatives, along with the ions for the cholesterol ring at *m/z* 369.

Figure 3 gives the full mass spectra for the cholesterol C_9 core aldehyde and its MOX derivative as obtained by ammonia CI. The aldehyde (Fig. 3A) gives ions for the ammonium adduct at *m/z* 558 and the cholesterol ring at *m/z* 369 and *m/z* 386. The ability to form the methoxime derivative supports the presence of an aldehyde function in these molecules. The ion at *m/z* 555 in Figure 3B is due to the loss of methanol from the methoxime ($[M + NH_4 - 32]^+$). The C_5 core aldehyde and its MOX derivative gave corresponding characteristic ions for the major peak. GLC examination of the TLC fractions corresponding to the diethyl acetals yielded peaks with retention times greater than those of the corresponding core aldehydes. These peaks showed signs of decomposition at the elevated temperatures necessary for steryl ester elution, and were not examined further. These TLC bands failed to yield the hydrazones or MOX derivatives. GLC analysis of the TLC band corresponding to the osmic acid ester of cholesteryl oleate diol was unstable during high temperature GLC, as indicated by the formation of variable peak proportions with retention times lower or greater than those of the parent cholesteryl oleate. The osmium tetroxide ester is an intermediate in the hydroxylation of polyunsaturated fatty acid esters and is not completely reduced with sodium bisulfite to the *cis* vicinal-diol. The presence of the diethyl acetals was attributed to their formation during the periodate cleavage of the fatty chain between the vicinal hydroxyl groups, since the reaction was carried out in 95% ethanol with HIO_4 being a strong acid.

Figure 4 shows the reverse-phase HPLC resolution of the DNPH derivatives of the C_5 and C_9 cholesteryl ester core aldehydes. A single peak is obtained for the C_9

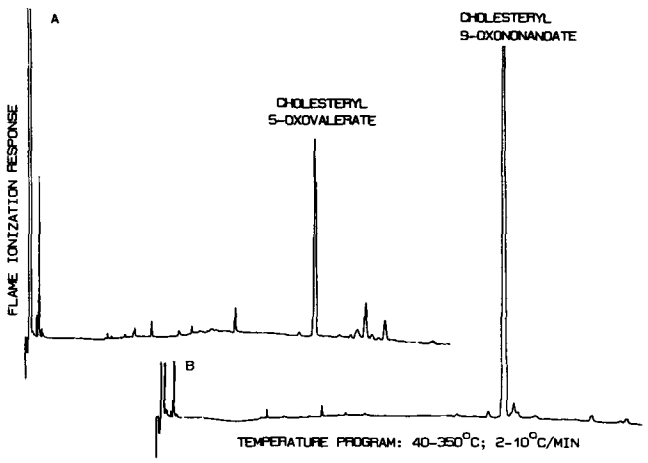

FIG. 2. Nonpolar capillary GLC separation of the C_5 (A) and C_9 (B) cholesteryl ester core aldehydes (free form) after recovery by TLC. GLC conditions, column (8 m × 0.30 mm); temperature program, 40–350°C; detector, FID.

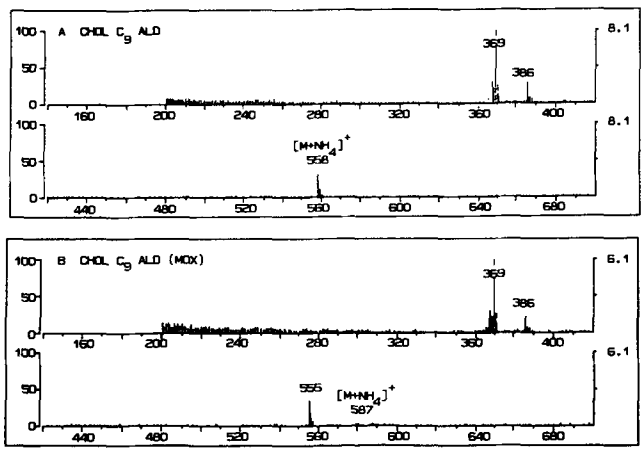

FIG. 3. Full mass spectra of cholesteryl 9-oxononanoate as obtained by GC/MS with ammonia CI. A, free aldehyde; B, methoxime (MOX). GC/MS conditions, nonpolar capillary column (8 m × 0.30); temperature program, as in Figure 2; mass spectra, GC/MS *via* direct capillary column inlet in the positive ammonia ionization mode.

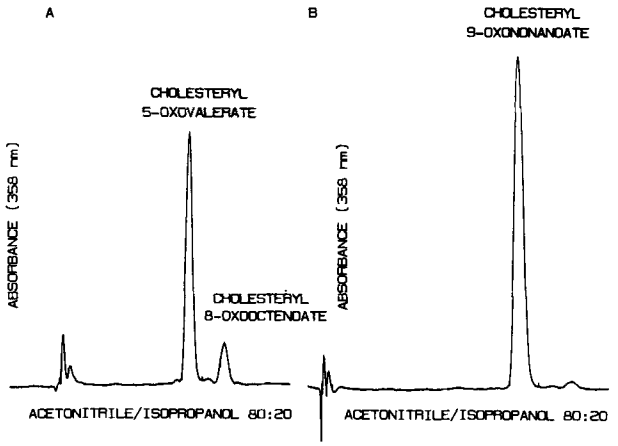

FIG. 4. Reverse-phase HPLC resolution of cholesteryl ester core aldehydes as the DNPH derivatives. A, 5-oxovalerate; B, 9-oxononanoate. HPLC conditions were as given in Materials and Methods. The retention time of the C_9 aldehyde was 15 minutes.

aldehyde, while the C_5 aldehyde gives also a minor peak for the 8:1 aldehyde, as established by LC/MS. Figure 5 shows the full negative CI mass spectra of the DNPH derivatives of the C_5 and C_9 cholesteryl ester core aldehydes. Only the molecular ions are seen. A correct molecular ion was also obtained for the 8:1 aldehyde among the oxidation products of the cholesteryl arachidonate. A GLC examination of the more polar oxidation products of the cholesteryl esters recovered near the origin of the TLC plate yielded peaks, which corresponded to cholesteryl esters with oxygenated sterol rings. The ring oxidation products obtained from the unsaturated fatty acid esters of cholesterol were identical to those derived from cholesteryl palmitate employed as a control. Saponification of these fractions revealed the presence of mixed cholestane triols, as well as other unidentified oxygen-containing sterols. On the basis of the total lipid profiles of the unsaturated cholesteryl ester oxidation products, the overall yield of the C_5 and C_9 core aldehydes was estimated to be about 10% (chromatograms not shown).

Characterization of diacylglycerol core aldehydes. The native and osmium tetroxide/periodic acid oxidized egg yolk PC were resolved by TLC using chloroform/methanol/water (65:35:6, vol/vol/vol) as solvent. Because of its greater polarity, the oxidized PC (R_f 0.26) was clearly resolved from the native PC (R_f 0.33) (chromatoplate not shown). Any residual PC, however, overlapped with the diethyl acetals of the PC-derived core aldehydes. There were minor fluorescent bands near the origin of the TLC plate, which were not further characterized. The identity of the core aldehydes was established by dephosphorylation of the PC-derived core aldehydes or their DNPH derivatives with phospholipase C followed by GC/MS or LC/MS.

Figure 6 shows the separation of the diacylglycerols containing the core aldehydes as the TMS and the MOX-TMS derivatives by nonpolar capillary GLC. Two major peaks are seen, which were shown by GC/MS to be due to the 9-oxononanoyl esters of palmitoyl and stearoylglycerols. The presence of the aldehyde function was indicated by the shift of both peaks to longer retention times following conversion to the MOX derivatives. Figure 7 gives the GC/EIMS spectra of the TMS ethers (Fig. 7A) of 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycerol and its MOX derivative (Fig. 7B). In neither instance was the molecular ion seen. However, readily detectable were the $[M - 15]^+$ ions due to loss of a methyl group from the TMS ethers. The *m/z* 145 ion represents $[CH(O)CHCH_2OSi(CH_3)_3]^+$, while the ion at *m/z* 129 represents $[CH_2CHCHOSi(CH_3)_3]^+$. The *m/z* 385 and 301 ions in Figure 8A are due to $[M - RCOO(C_9ALD)]^+$ and $[M - RCOO(C_{16})]^+$, respectively, while the *m/z* 229 and 313 ions are due to

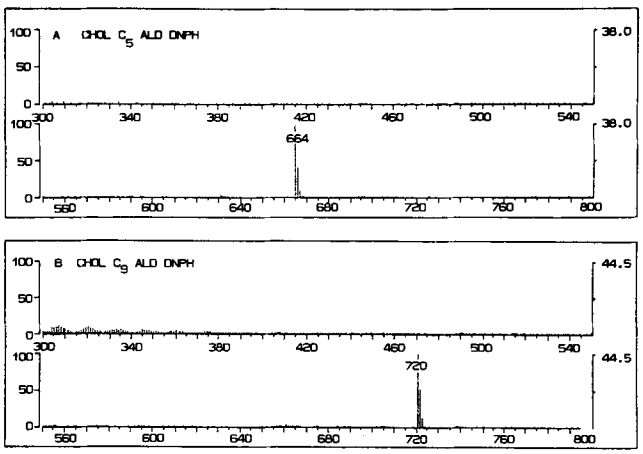

FIG. 5. Full mass spectra of cholesteryl ester core aldehyde DNPH derivatives as obtained by reverse-phase LC/MS with negative CI. A, 5-oxovalerate; B, 9-oxononanoate. LC/MS conditions were as given in Materials and Methods.

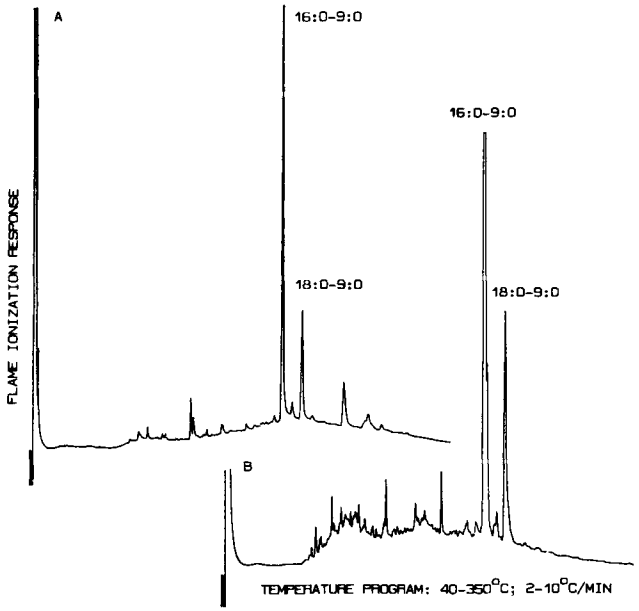

FIG. 6. Nonpolar capillary GLC profiles of diacylglycerol moieties of oxidized egg yolk PC containing core aldehydes. A, TMS ethers; B, MOX-TMS ethers. GLC conditions were as in Figure 2.

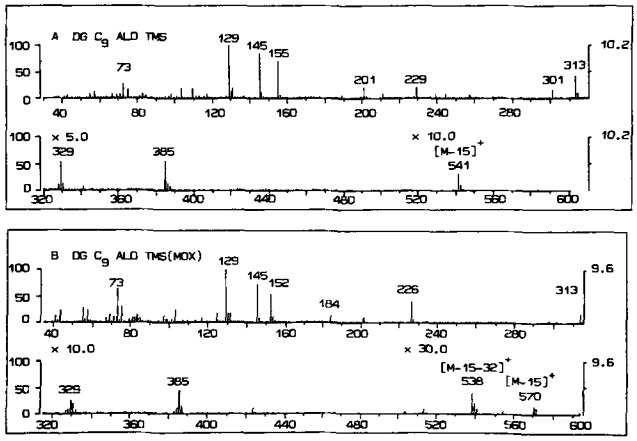

FIG. 7. Full mass spectra of 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycerol as obtained by GC/MS with EI ionization. A, TMS ether; B, MOX-TMS ether. GC/MS conditions as in Figure 2.

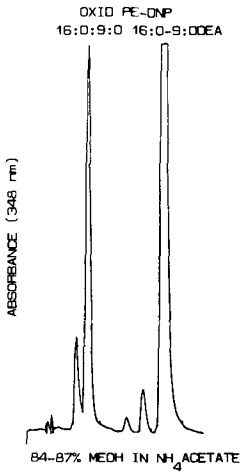

FIG. 8. Reverse-phase HPLC resolution of 1-palmitoyl-2-(9-oxononanoyl)-GPE-DNP; 16:0-9:0, C_9 core aldehyde of PE DNP; 16:0-9:0 DEA, diethyl acetal of C_9 core aldehyde of PE DNP. HPLC conditions as given in Materials and Methods. The retention time of the 16:0-9:0 diethyl acetal was 10 minutes.

$[RCO(C_9ALD) + 74]^+$ and $[RCO(C_{16}) + 74)]^+$, respectively. The *m/z* 155 is due to $[RCO(C_9ALD)]^+$. The *m/z* 538 ion in Figure 7B is due to loss of methanol from the MOX group in $[M - 15]^+$, while the *m/z* 385 and 329 ions are due to $[M - RCOO(C_9ALDMOX)]^+$ and $[M - RCOO(C_{16}) - H]^+$, respectively. The *m/z* 226 and 313 ions are due to $[RCO(C_9ALDMOX) - CH_3OH + 74]^+$ and $[RCO(C_{16}) + 74]^+$, respectively. The *m/z* 184 and *m/z* 152 ions are due to $[RCO(C_9ALDMOX)]^+$ and $[RCO(C_9ALDMOX) - CH_3OH]^+$ fragments, respectively. Two peaks were seen also in the LC/NCIMS (NCI, negative chemical ionization) profile of the DNPH derivatives of the diacylglycerol core aldehydes prepared from the oxidized egg yolk PC. The single negative ions produced for each peak corresponded to the $[M]^-$ of palmitoyl-9-oxononanoyl and stearoyl-9-oxononanoylglycerol DNPH (chromatograms and mass spectra not included). The total ion current profile was similar to that of the UV absorption profile, while the peak ratios corresponded to the ratios of the 16:0/18:0 fatty acids in the egg yolk PC from which the oxidation products were derived.

Characterization of GPE core aldehyde. Osmium tetroxide/periodic acid oxidation of the DNP derivative of synthetic 16:0-18:1 GPE gave two bands on TLC: one corresponding mainly to the core aldehyde (R_f 0.53) and another one (R_f 0.60) corresponding to the diethyl acetal of the core aldehyde. Figure 8 shows the reverse-phase HPLC profile of the diethyl acetal band which was cross-contaminated with small amounts of the core aldehyde. The major peaks were separately collected and examined by FAB-MS.

Figure 9 shows the pertinent portions of the FAB-MS spectra of the core aldehyde esters containing the free aldehyde and the diethyl acetal functions. The ion *m/z* 796 in Figure 9A represents $[M + Na]^+$ of the free aldehyde molecule, while the *m/z* 870 and 865 ions in Figure 9B represent the $[M + Na]^+$ and $[M + NH_4]^+$, respectively. The *m/z* 824 ion is due to loss of ethanol from the diethyl acetal group of $[M + Na]^+$, while the *m/z* 802 ion is due to $[M + H - C_2H_5OH]^+$. The presence of the DNP group on the ethanolamine moiety of GPE allowed the UV monitoring of the presence of non-aldehyde peaks formed as minor byproducts in the oxidation reaction. These were considered to be the acids and their ethyl esters of the core aldehydes, but were not further identified. On the basis of the reverse-phase HPLC profiles of the total reaction mixture, the overall yield of the C_5 and C_9 core aldehydes of the DNP-PE was estimated at about 20% of the unsaturated starting material.

In conclusion, this study reports the synthesis and characterization of 5-oxovaleric and 9-oxononanoic acid esters of cholesterol and the common GPL, PC and PE. The core aldehydes are characterized by chromatographic

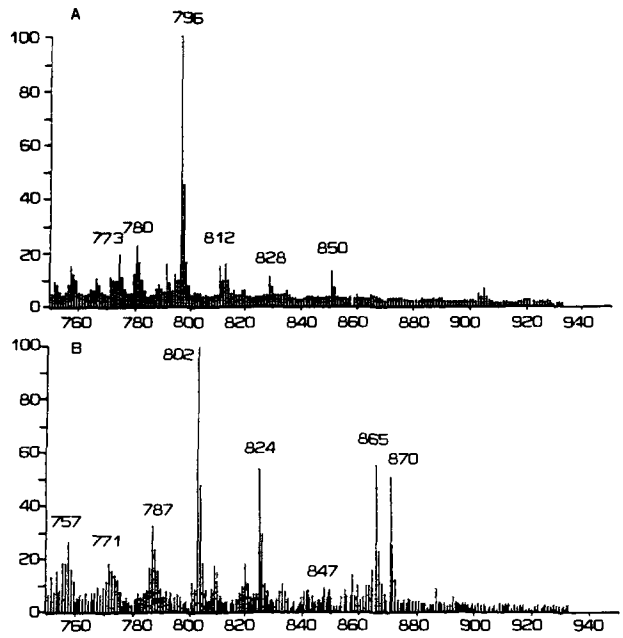

FIG. 9. FAB-MS spectra of 1-palmitoyl-2-(9-oxononanoyl)-GPE-DNP. A, free aldehyde; B, diethyl acetal. FAB-MS conditions were as given elsewhere (18).

and mass spectrometric methods in the form of various derivatives, which clearly distinguish them from closely related byproducts formed during synthesis. The semisynthetic lipid ester core aldehydes provide reliable standards for the isolation and identification of the common lipid ester core aldehydes among the secondary peroxidation products of natural cholesteryl esters and glycerolipids.

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Seawater Fatty Acids and Lipid Classes in an Urban and a Rural Nova Scotia Inlet[1]

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Seawater samples collected in the summer of 1989 in Nova Scotia inlets were analyzed for lipid content to examine water quality. One inlet, the Northwest Arm, is located adjacent to an urban center, while the other, Ship Harbour, is located in an uninhabited area and contains three commercial mollusk farms. Lipids in the dissolved and particulate fractions were measured by Chromarod-Iatroscan thin-layer chromatography with flame ionization detection and by gas chromatography. Samples collected near the urban center had higher levels of particulate hydrocarbons (28 ± 15 μg/L) than those collected in the relatively pristine environment (11 ± 1 μg/L). The urban center samples also had higher levels of particulate free aliphatic alcohols (14 ± 5 *vs.* 8 ± 1 μg/L) and free fatty acids (5 ± 1 *vs.* <0.5 μg/L), suggesting degradation of wax esters and other fatty acid esters. Dissolved and particulate matter fatty acids contained a higher proportion of monounsaturated acids near the urban center (33–35 *vs.* 25–29% of the total fatty acids). The essential fatty acids 20:5n-3 (eicosapentaenoic acid) and 22:6n-3 (docosahexaenoic acid), presumably of algal origin, were more prominent in the pristine environment (up to 3.5%), where mollusk aquaculture is practiced. Fatty acid markers of toxic algae were virtually absent (<0.2%) in samples taken from both locations.
Lipids 27, 651–655 (1992).

Lipids are present in seawater in organisms, in detritus, and in the operationally defined "dissolved" fraction. Lipid class data from extracts of dissolved and particulate matter are useful as indicators of pollution, certain types of organisms, or of anabolic or catabolic processes (1), while certain fatty acids may be used as marker or signature compounds for specific organisms (2,3). Markers of particular interest in eastern Canada are the polyunsaturated fatty acids associated with marine dinoflagellates (2) and the marine diatom *Nitzschia pungens* (4). Certain dinoflagellates have long been known to cause paralytic shellfish poisoning in this region, while *Nitzschia pungens* was the source of domoic acid present in cultivated blue mussels which caused a recent outbreak of amnesic shellfish poisoning here (5,6). There is currently a global increase in reports of such catastrophic blooms; areas affected include polluted coastal waters and aquaculture sites (7). Thus we measured fatty acids and lipid classes in seawater samples collected from two such areas represented by two inlets on the coast of Nova Scotia in eastern Canada. One inlet, the Northwest Arm, is surrounded by a city; the other, Ship Harbour, is located in a relatively uninhabited area used for blue mussel farming.

MATERIALS AND METHODS

Seawater samples. Samples from the Northwest Arm, a small marine inlet about 5 km long surrounded by the City of Halifax, were collected in the Aquatron Laboratory of Dalhousie University. Water was pumped from a depth of about 12 m in the arm, through an underground fiberglass pipeline to the Aquatron Laboratory. Samples of unfiltered water were collected around noon on July 17, 21 and 25 and on August 2, 1989. The average water temperature over the four sampling days was 12 ± 3°C.

Samples were also collected on August 3, 1989 from Ship Harbour, a marine inlet located about 80 km east of Halifax. Ship Harbour is about 8 km long, and is surrounded by six freshwater lakes from which five rivers drain into various points of the harbor. Ship Harbour is the site of three farms for blue mussels, *Mytilus edulis*; it has no major settlements or heavy industry located on its shores. Samples were taken around 3 p.m. at 0.5 m, near the center of the harbor. The water temperature was 21°C.

Chlorophyll *a* was determined in a Turner fluorometer according to Strickland and Parsons (8). Salinity was measured using a Guildline Autosal (Smiths Falls, Ontario, Canada) and the nutrients were run on a Technicon Autoanalyser II (Tarrytown, NY).

Lipid class analyses. Dissolved and particulate lipid classes were measured as described previously (9). Briefly, samples collected in precleaned and sample-rinsed amber glass solvent bottles were screened through a 75 μm metal sieve, then filtered onto precombusted Gelman A/E glass fiber filters (Gelman Sciences, Ann Arbor, MI). After addition of the internal standard, *n*-octadecan-3-one, "dissolved" lipids were extracted with dichloromethane and "particulate" lipids (see Discussion) were extracted with dichloromethane/methanol (2:1, vol/vol). The extracts were washed with distilled water to remove non-lipid material, dried over anhydrous sodium sulfate, and concentrated under nitrogen. The concentrated lipid extracts were spotted onto Chromarods-SIII for thin-layer chromatographic separation of lipid classes. Samples were focused (10) into a narrow band on the silica gel surface by developing to the origin in acetone, and then lipid classes were separated in a step-wise procedure using developing systems of increasing polarity. Neutral lipids were separated in two solvent systems containing hexane, diethyl ether and formic acid, 99:1:0.05 (vol/vol/vol) followed by 80:20:0.1. Polar lipids were then separated into "acetone-mobile polar lipids" and phospholipids using 100% acetone and a mixture of chloroform, methanol and water (65:35:4). Lipid classes were measured by flame ionization detection in an Iatroscan MK III (Newman-Howells Assoc., Wales, U.K.); the signal was recorded on a Spectra-Physics computing integrator (SP 4270;

[1]O.S.C. contribution 173.

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Abbreviations: br, branched-chain; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FA, fatty acid; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry.

Spectra-Physics, St. Albans, U.K.). They were quantified using calibration curves obtained from scans of authentic standards (Sigma, St. Louis, MO).

Fatty acid analyses. Fatty acids in the August samples were measured by gas chromatography (GC) as methyl esters (11). Separations were performed on a fused silica capillary column (30 m × 0.32 mm i.d.) coated with Supelcowax-10 (Supelco Inc., Bellefonte, PA) in a Perkin-Elmer Model 8420 gas chromatograph (Perkin-Elmer, Norwalk, CT). Fatty acids were identified by comparing their retention times, both before and after catalytic hydrogenation over PtO_2, with those of standard mixtures of well-established composition. Some identities were also confirmed by GC/mass spectrometry (MS) using a Finnigan MAT (San Jose, CA) Ion Trap detector (12).

RESULTS

The temperature and salinity data (Table 1) from the two Nova Scotia inlets suggest a significant oceanic contribution to the source of the seawater samples from the Northwest Arm and a significant freshwater contribution to the upper water layer of Ship Harbour. The salinity of the Northwest Arm water was greater than that measured throughout the water column in nearby Bedford Basin during the spring or summer (13,14). Bedford Basin, which is normally described as a marine inlet, is connected to the Northwest Arm *via* Halifax Harbour.

TABLE 1

Salinity, Nutrients and Chlorophyll *a* in Nova Scotia Inlets in August 1989

	Northwest Arm[a]	Ship Harbour[b]
Salinity (‰)[c]	31.65 ± 0.05	18.42 ± 1.19
Phosphate (μmol/L)	0.99 ± 0.04	0.24 ± 0.02
Nitrate (μmol/L)	0.99 ± 0.02	0.17 ± 0.03
Chlorophyll *a* (μg/L)	0.26 ± 0.12	0.24 ± 0.03

[a]Mean ± SD of three separate samples collected on August 2.
[b]Mean ± SD of three separate samples collected on August 3.
[c]Parts per thousand.

Both the nutrient and chlorophyll *a* concentrations were low in the two inlets suggesting post-algal bloom conditions; they were similar to those measured in Bedford Basin after spring blooms (15) though the nitrate and chlorophyll *a* concentrations (Table 1) were even lower than post-bloom in Bedford Basin. Chlorophyll *a* concentrations (Table 1) were also lower than measured during the summer in Bedford Basin (13,16).

Total particulate lipids present in the Northwest Arm samples were at a concentration of 156 ± 59 μg/L (mean ± SD, n = 4). Total dissolved lipids were present at a level of 87 ± 33 μg/L over the four-week sampling period. There was no continuous trend in the total lipid data for the four sampling days.

The average levels of total dissolved and particulate lipids in Ship Harbour were 156 μg/L and 83 μg/L, respectively, in the August samples. The individual lipid class concentrations for Ship Harbour were generally similar to those measured in the Northwest Arm samples (Table 2), except in the case of particulate hydrocarbons, free fatty acids and free aliphatic alcohols which were lower in Ship Harbour, and dissolved free fatty acids and acetone-mobile polar lipids which were higher.

Detailed GC and GC/MS analyses of the total fatty acids in the August samples revealed the presence of 33–40 identifiable fatty acids (Table 3). Particulate lipids from the Northwest Arm showed the widest spectrum of fatty acids, suggesting a greater variety of sources. GC/MS analyses also indicated that several hydrocarbons and phthalates of unknown origin occurred in the concentrates.

DISCUSSION

Representatives from as many as 16 lipid classes have been found in the dissolved and particulate fractions of seawater (1). These fractions are obtained by firstly passing samples through a screen (50–250 μm mesh) to remove larger zooplankton, and then by filtering through glass-fiber filters (0.7–1.2 μm pore size) to separate dissolved and particulate material. Lipids present in dissolved and particulate matter in coastal waters are derived from various

TABLE 2

Dissolved and Particulate Lipid Class Concentrations (μg/L ± SD) in Nova Scotia Inlets During the Summer of 1989

	Northwest Arm[a]		Ship Harbour[b]	
	Diss.	Part.	Diss.	Part.
Hydrocarbons	11 ± 6	28 ± 15	12 ± 4	11 ± 1
Wax and steryl esters	9.1 ± 5.1	10 ± 3	10 ± 1	11 ± 1
Triacylglycerols	14 ± 6	12 ± 3	27 ± 4	12 ± 0.2
Free fatty acids	12 ± 5	14 ± 5	21 ± 2	7.6 ± 1.3
Free aliphatic alcohols	2.5 ± 1.9	5.2 ± 3.9	trace[c]	trace
Sterols	2.6 ± 1.8	3.5 ± 1.6	4.1 ± 1.9	2.5 ± 0.3
Acetone-mobile polar lipids[d]	42 ± 26	70 ± 35	79 ± 9	34 ± 8
Phospholipids	3.3 ± 1.9	8.4 ± 6.8	3.2 ± 0.6	5.0 ± 0.7

[a]Data are mean ± SD of four sampling days between July 17 and August 2, 1989.
[b]Data are mean ± SD of three separate samples collected on August 3, 1989.
[c]<0.5 μg/L.
[d]Includes monoacylglycerols, glycolipids and chlorophylls.

TABLE 3

Fatty Acid Composition (wt%) of Lipid Extracts of Dissolved and Particulate Material from Nova Scotia Inlets in August 1989

	Northwest Arm		Ship Harbour	
Fatty acid	Diss.	Part.	Diss.	Part.
12:0[a]	3.6	1.3	1.1	4.1
13:0	0.9	1.4	0.8	0.4
Iso-14:0	1.2	1.3	—	1.1
14:0	8.6	7.6	8.9	11.5
Iso-15:0	1.7	1.4	1.0	1.3
Anteiso-15:0	1.4	1.9	1.7	1.3
15:0	4.1	3.9	3.7	2.9
Iso-16:0	1.0	1.0	1.0	0.7
Pristanate	1.0	1.0	1.0	0.9
7Me 16:0[b]	0.7	0.8	—	0.6
16:0	26.6	24.1	22.9	26.3
Anteiso-17:0	trace	trace	—	—
17:0	1.2	1.2	1.1	1.0
18:0	7.2	5.3	8.9	12.9
20:0	—	0.5	0.5	—
22:0	0.6	—	0.8	0.8
23:0	0.4	0.3	3.5	—
24:0	0.9	3.2	2.9	1.6
Total saturated fatty acids (FA)	61.1	56.2	59.8	67.4
14:1	5.2	4.5	4.5	3.5
15:1	2.2	1.9	3.2	2.2
16:1n-9[c]	—	0.3	—	0.3
16:1n-7	11.8	12.8	7.9	9.4
16:1n-5	0.4	0.6	0.8	0.6
18:1n-9	11.7	10.5	9.2	6.5
18:1n-7	1.3	1.8	1.2	1.5
18:1n-5	—	0.6	0.3	0.4
20:1n-11	—	0.4	—	—
20:1n-9	—	0.3	0.4	—
20:1n-7	—	0.3	—	—
22:1n-11 plus n-13	0.3	—	0.6	0.4
22:1n-9	0.5	—	0.5	—
22:1n-7	—	—	0.2	—
24:1	—	1.0	—	—
Total monoenoic FA	33.4	35.0	28.9	24.8
16:2n-6	0.5	0.6	0.7	0.5
18:2Δ5,11[d]	0.2	—	—	—
18:2n-7	0.7	—	0.4	—
18:2n-6	1.4	1.7	1.6	1.2
20:2n-6	—	0.3	—	—
Total dienoic FA	2.8	2.6	2.7	1.7
16:3n-4	1.4	1.7	0.4	0.9
18:3n-6	—	—	—	0.3
18:3n-4	—	0.2	—	—
18:3n-3	0.3	0.6	0.6	0.9
20:3n-3	—	0.3	2.5	—
Total trienoic FA	1.7	2.8	3.5	2.1
16:4n-3	—	—	—	0.3
16:4n-1	0.2	—	—	—
18:4n-3	—	0.8	0.7	0.8
20:4n-6	—	0.1	—	—
20:4n-3	0.1	—	—	—
22:4n-3	0.3	—	—	—
Total tetraenoic FA	0.6	0.9	0.7	1.1
20:5n-3	0.5	1.2	1.4	1.3
22:6n-3	0.3	1.2	3.5	1.9

[a]Ratio of fatty acid carbon atoms to carbon-carbon double bonds.

[b]Methyl group on C_7.

[c]n Denotes the position of the nearest double bond to the methyl end of the fatty acid.

[d]Bond position relative to the carboxyl group.

sources (17,18). Biogenic and anthropogenic inputs of such compounds occur directly in aquatic environments (15) or they are transported from further afield by ocean currents (19), by rivers (20) or in airborne particulate matter (21).

We compare here dissolved and particulate lipid data from two inlets where sources for the lipids are likely to be different. We also compare these data with published data from another inlet, Bedford Basin, located on the same coast. Bedford Basin has been the site of several studies involving lipid analyses (9,13-16,22). By contrast with these previous studies, in this study we measured not only the complete suite of lipid classes in both dissolved and particulate matter, but also the complete spectrum of fatty acids in these two fractions.

The concentrations of particulate lipids in the Northwest Arm were similar to those measured near the center of Bedford Basin after the spring bloom (15,22). The concentrations of dissolved lipids, however, were lower than measured during bloom periods in Bedford Basin (15) where dissolved lipids were usually present at higher concentrations than were the particulate lipids. It is possible that sampling the Northwest Arm water through a pipeline had in some way upset the natural balance between dissolved and particulate lipids, although the fear had been that dissolved lipid concentrations would be biased upward as a result of cell lysis.

The lack of a trend in the Northwest Arm total lipid data extended to the time series data for all but one of the lipid classes. For this reason, all the Northwest Arm lipid class data have been averaged in order to more easily compare with the Ship Harbour lipid data (Table 2). Such a grouping seems reasonable as the week to week variability in the Northwest Arm data was close to the sample to sample analytical variability for Ship Harbour. The standard deviation for the Northwest Arm data is similar to that obtained from triplicate Ship Harbour samples, though it is larger for the time series in all but one case (Table 2).

The hydrocarbon figures for Ship Harbour were similar in magnitude to the 10 μg/L of C_{14}-C_{21} hydrocarbons measured in nearby Jeddore Harbour (23). High levels of branched-chain (br) hydrocarbons in Jeddore Harbour were thought to have been derived from diesel fuels. These hydrocarbons are, however, normal constituents of blue-green algae (24), and a strong summer input of complex hydrocarbons from algae cannot be ruled out. The origins of the Ship Harbour hydrocarbons (Table 2) may thus be quite diverse. The average particulate hydrocarbon concentration measured in the Northwest Arm samples is not only higher than measured in Ship Harbour, but it is also higher than averages measured in Bedford Basin (14,15) and much higher than averages measured over the Scotian Slope (14). Urban runoff is a possible contributor to these high total particulate hydrocarbon levels (20).

Free fatty acids, free aliphatic alcohols and acetone-mobile polar lipids were present at different concentrations in the Ship Harbour and Northwest Arm samples. Such classes, which contain indicators of lipid degradation (1), might be expected in a post-bloom situation. High concentrations of particulate wax and steryl esters (Table 2) also might be expected in this situation; the levels of this zooplankton indicator class (1) were the same in the Northwest Arm and Ship Harbour samples, but were

higher than over the Scotian Slope or in Bedford Basin (14,15). It is not clear why certain degradation indicators were more prominent in one fraction in one location than in the other, although it is likely that such differences are related to anthropogenic inputs to the Northwest Arm.

The oceans are a repository for many fatty acids because of the multitude of marine flora and fauna. The presence of the long-chain saturated fatty acids 19:0 and 20:0 in a Newfoundland bay has been noted (25). Here we show that 22:0, 23:0 and 24:0 are also present in marine dissolved or particulate matter (Table 3). Such saturated fatty acids may originate in crustacean faecal matter (26). The proportions of branched-chain C_{15} fatty acids were 3–5 times higher in the Nova Scotian inlets (Table 3) by comparison with the Newfoundland bay (25). Such fatty acids are believed to be of bacterial origin and it is notable that the levels of odd carbon number branched-chain fatty acids tend to be higher in the samples taken from the urban inlet (Table 3). The levels of these fatty acids are more similar to those found in Jeddore Harbour, Nova Scotia where br C_{15} and br C_{17} were in the range 0.6–1.7 wt% in the August sample (23). The high levels of unbranched 15:0 (2.9–4.1 wt%) in dissolved and particulate material (Table 3) compare well with previous measurements of 15:0 (23,25). This saturated fatty acid is easily identified and quantified and, because it is ubiquitous in marine phytoplankton (27), it may be a useful indicator of algal blooms.

In contrast to C_{15} fatty acids, the even-chain saturated fatty acids 14:0, 16:0 and 18:0 are found at significant levels in most marine samples. It is thus not surprising that these unbranched fatty acids contribute significantly to the total fatty acid pool in both dissolved and particulate matter from both locations (Table 3). The levels, in μg/L, of these fatty acids are also likely to be similar. The total levels of the acyl lipid classes where fatty acids are a major component of the lipid structure (wax and steryl esters, triacylglycerols, free fatty acids and phospholipids) are in the range 36–61 μg/L. Thus, the levels of the major saturated fatty acids are likely to be similar in the dissolved and particulate samples from each location. This suggests a ubiquitous source, presumably algae.

The monoethylenic fatty acids include 14:1 at about half the level of 14:0. In lipids extracted from most marine animals, 14:1 is a very minor component (<0.5% of the fatty acids) and its presence in seawater is not clearly explainable. The 16:1 and 18:1 fatty acids are also ubiquitous and not remarkable qualitatively or quantitatively; however, 20:1 and 22:1 are usually associated with copepod lipids (25,28) and were not previously found in the coastal seawater sample that had been screened to remove zooplankton (25). These long-chain monoene fatty acids were also absent from suspended particulate matter collected at oceanic stations (29), but they were present in particulate matter collected in Bedford Basin after screening through 163 μm bolting cloth (16). Bedford Basin is connected to the Northwest Arm, thus the source of the 20:1 and 22:1 is probably the same in the two studies and may be related to high wax and sterol ester levels (Table 2).

The polyunsaturated fatty acids are all minor constituents, although the Ship Harbour dissolved lipid sample had rather higher quantities of 20:5n-3 (eicosapentaenoic acid—EPA) and 22:6n-3 (docosahexaenoic acid—DHA). The level of these polyenoic acids was also slightly higher in the particulate matter from Ship Harbour. The significant levels of these labile fatty acids, which are abundant in marine phytoplankton (27), suggest that an algal bloom was just decaying in Ship Harbour. The high concentration of dissolved free fatty acids in this water (Table 2) would support this contention. Whether this had already taken place in the Northwest Arm is difficult to assess because of the deeper water source. After EPA and DHA, the most important polyenes were 18:2n-6 and 16:3n-4 (Table 3). Both these fatty acids could have been synthesized by any of several phytoplankton common in these waters. Two polyunsaturated fatty acids that are notable, at least from a public health perspective, because of their virtual absence from the extracts are 18:5n-3 and 16:4n-1. The first is a marker for marine dinoflagellates (2) which has been reported to be an ichthyotoxin (30), while the second is a major contributor to the fatty acids of the toxic marine diatom *Nitzschia pungens* (4). These seawater data thus provide a baseline showing a normal fatty acid and lipid class distribution against which samples taken during catastrophic blooms could be compared. Good baseline data for coastal areas are important in view of the global increase in geographic extent and variety of toxic algal blooms (7).

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ARTICLE

Studies on the Transfer of Tocopherol Between Lipoproteins

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The net transfer of labeled α-tocopherol from donor to acceptor lipoproteins at physiological concentrations was investigated. Labeled lipoproteins were isolated i) following *in vitro* addition of [3,4-^{3}H] *all rac*-α-tocopherol to plasma, or ii) from plasma obtained 12–16 h after ingestion by normal subjects of an oral dose (100 mg each) of 2*R*,4′*R*,8′*R*-α-[5,7-(C^2H_3)$_2$]tocopheryl acetate and 2*S*,4′*R*,′R-α-[5-C^2H_3]tocopheryl acetate. A constant amount (on a protein basis) of labeled lipoprotein was incubated with an increasing amount of unlabeled acceptor lipoprotein for 2 h at 37°C. No discrimination between stereoisomers of α-tocopherol was detected. Labeled VLDL and labeled LDL (very low and low density lipoproteins, respectively) tended to retain their labeled tocopherol. Labeled high density lipoproteins (HDL) readily transferred the labeled tocopherol to VLDL (>60% transferred), while the transfer to LDL was dependent upon the ratio of labeled HDL/LDL with a lower net transfer at higher ratios. This dependency of the distribution of tocopherol upon the ratio of HDL/LDL was also observed *in vivo*. The tocopherol/mg HDL protein was measured in 11 subjects with varying HDL levels. As the % HDL in the plasma increased from 14 to 50%, the tocopherol/HDL protein also increased (r^2 = 0.37, $P < 0.05$).
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Vitamin E absorption and transport has been studied using deuterated tocopherols. The ratio of deuterated/total α-tocopherol following an oral dose of the labeled material to humans peaks first in chylomicrons, then in very low density lipoproteins (VLDL), and then simultaneously in low and high density lipoproteins (LDL and HDL, respectively) (1). Studies of the discrimination between tocopherols have demonstrated that there is a specific mechanism in the liver for the incorporation of *RRR*-α-tocopherol into VLDL (2–4), and that this mechanism is essential for the maintenance of normal plasma α-tocopherol concentrations (5). However, we have also found that independent of this specific mechanism there is transfer of tocopherols to LDL and HDL during catabolism of the triglyceride-rich lipoproteins. This latter transfer mechanism is sufficient to maintain normal plasma levels of α-tocopherol, when large doses of vitamin E are given to patients with familial isolated vitamin E deficiency, who do not have normal secretion of α-tocopherol in VLDL (5).

The present paper describes a series of *in vitro* incubation studies using lipoproteins labeled with radioactive or deuterated tocopherols then mixed and incubated with unlabeled lipoproteins to determine whether tocopherol transfers between lipoproteins in the absence of other mediators. Previously, Massey (6) has studied the transfer of tocopherol and cholesterol between lipoproteins and liposomes and demonstrated that cholesterol transfers more readily than does tocopherol. Kayden and Bjornson (7) and Bjornson *et al.* (8) demonstrated that tocopherol readily exchanges between lipoproteins and erythrocytes, and that the order of the rate of exchange was HDL > LDL > VLDL. Granot *et al.* (9) have demonstrated that tocopherol transfer between lipoproteins is not assisted by neutral lipid transfer protein. All of these studies have employed the use of lipoproteins labeled *in vitro* with radioactive tocopherol. Potentially, this label might not equilibrate with the lipoproteins similarly to metabolically incorporated tocopherols. Therefore, we first devised our experiments using lipoproteins isolated from serum incubated with radioactive tocopherol, then we verified our results using lipoproteins isolated from plasma obtained 12–16 h following the oral administration of deuterated tocopherols.

MATERIALS AND METHODS

Radioactive tocopherol. Before use, [3,4-^{3}H] *all rac*-α-tocopheryl acetate (a gift from Dr. W. Cohn, Hoffmann La Roche, Basel, Switzerland) was subjected to alkaline hydrolysis in the presence of excess ascorbic acid followed by extraction with hexane. The [^{3}H]α-tocopherol was then used immediately for the labeling of plasma (described below). More than 95% ^{3}H was found to coelute with an authentic sample of added unlabeled *all rac*-α-tocopherol in high-performance liquid chromatography (HPLC) (10).

To label the plasma with [^{3}H]tocopherol, a modification of the method used by Goodman *et al.* (11) for labeling plasma with radioactive cholesterol was devised. Approximately 15 μCi of [^{3}H]tocopherol in hexane was dried under nitrogen and immediately resuspended in acetone (200 μL). The sample was mixed vigorously with a vortex mixer, then was taken up into a syringe and forcibly injected under the surface of approximately 11 mL of plasma in a scintillation vial. The vial was flushed with nitrogen, sealed and incubated in the dark at room temperature overnight. The vial was then flushed with nitrogen for 1 h to allow the acetone to evaporate. The lipoproteins (VLDL, LDL and HDL) were isolated by the micro-method of lipoprotein isolation described below.

Deuterated tocopherols. The syntheses and analysis of deuterated α-tocopherols have been described previously (12,13). Drs. Keith Ingold and Graham Burton (National Research Council, Ottawa, Ontario, Canada) provided the tocopherols labeled with deuterium and carried out the analyses. An oral dose containing 100 mg of each 2*R*, 4′*R*,8′*R*-α-[5,7-(C^2H_3)$_2$]tocopheryl acetate, a source of hexadeuterated α-tocopherol with natural stereochemistry, and 2*S*,4′*R*-α-[5-C^2H_3]tocopheryl acetate, a source of trideuterated α-tocopherol with unnatural stereochemistry, was consumed by each subject. 2-*Ambo*-α-[5,7,8-(C^2H_3)$_3$] tocopherol (d_9-tocopherol), used as an internal standard, was added in known amount to each plasma, or lipoprotein sample, immediately prior to lipid extraction (1,14). The lipid extracts were purified by passage through an

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; GC/MS, gas chromatography/mass spectrometry; HDL, high density lipoproteins; HPLC, high-performance liquid chromatography; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

analytical high-performance silica gel column. The amounts of d_3-, d_6-, d_9- and unlabeled (d_0) α-tocopherols in the tocopherol fractions collected were determined by gas chromatography/mass spectrometry (GC/MS) after conversion to their trimethylsilyl ethers (12,14). The absolute concentrations of d_0-, d_3- and d_6-α-tocopherols were obtained by comparing the respective peak areas with the peak area of the added d_9-α-tocopherol, as described previously (1,2,5,12,14).

Subjects. This study was carried out with the approval of the Institutional Review Board of New York University Medical Center. Three normal control subjects gave written informed consent and had no abnormalities of lipid or lipoprotein metabolism. These subjects consumed both deuterated tocopherols with the evening meal, then a blood sample (150 mL) was drawn into 0.05% ethylenediaminetetraacetic acid (EDTA) on the morning of the following day. The plasma was separated from the erythrocytes by centrifugation and immediately used for the isolation of lipoproteins labeled with deuterated tocopherols. Plasma for the isolation of unlabeled lipoproteins was obtained from the New York Blood Center (New York, NY).

Eleven subjects participated in the analysis of the distribution of α-tocopherol and cholesterol in lipoproteins. These subjects had a variety of abnormalities of lipoprotein metabolism which resulted in different levels of HDL cholesterol (Table 1). Following an overnight fast, two blood samples were collected; one serum sample for the analysis of cholesterol and HDL cholesterol by the clinical laboratory, and one plasma sample for the isolation of lipoproteins by the micro-method described below and the measurement of tocopherol by HPLC (10).

Lipoprotein isolation. Two separate protocols were used to isolate lipoproteins by ultracentrifugation: i) a macro-method was used to isolate the lipoproteins from the plasma prior to incubation; and ii) a micro-method was used to isolate the lipoproteins following incubation, or from small volumes of plasma. A precipitation technique was also used to re-isolate lipoproteins following incubation, as described below.

In the macro-method the lipoproteins were isolated by sequential ultracentrifugation by the method of Havel *et al.* (15) using a 50.2 Ti rotor in a L5-65 Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 100,000 × *g* for 4 h for isolation of VLDL and LDL and for 48 h for isolation of HDL with plasma adjusted with solid KBr to the following densities: VLDL ($d < 1.019$), LDL ($1.019 < d < 1.063$) and HDL ($1.063 < d < 1.21$). The lipoprotein fractions were dialyzed extensively against saline (0.15 M NaCl, 0.3 mM EDTA, pH 7.4), were filtered with a 0.45 μm^2 sterile filter unit (Nalge Co., Rochester, NY), the protein contents were determined by the method of Lowry *et al.* (16) with the addition of diethyl ether to remove lipids from the aliquots used for analysis. The lipoprotein fractions were stored at 4°C for less than two weeks prior to the incubation studies.

In the micro-method of lipoprotein isolation a TL 100 Beckman ultracentrifuge was used to isolate the indicated lipoprotein fractions by centrifugation at 435,680 × *g* using a fixed angle rotor (TLA 100.2) with density changes achieved by adding solid KBr. For isolation of lipoproteins for incubations, the densities above were used with a 2 h centrifugation to isolate VLDL, or LDL, and a 4 h centrifugation to isolate HDL. The lipoprotein fractions were dialyzed, and the protein contents were determined as described above. The labeled lipoproteins were filtered using a sterile 0.45 μm^2 syringe filter (Gelman Sciences, Ann Arbor, MI), were stored at 4°C and used within two days of isolation.

TABLE 1

Characteristics and Plasma Tocopherol and Cholesterol Concentrations of Subjects[a]

		Tocopherol		Cholesterol	
Subject	Sex	α (μg/mL)	γ (μg/mL)	mg/dL	% HDL
1	f	16.4	1.1	270	14.3
2	m	14.0	1.3	275	14.4
3	m	10.9	1.9	279	15.4
4	m	12.7	1.1	269	17.7
5	m	5.4	0.6	217	17.9
6	m	8.8	1.3	195	35.4
7	f	22.2	0.4	183	36.2
8	f	24.0	1.3	237	38.4
9	m	16.7	0.8	263	39.1
10	m	14.9	1.6	254	44.6
11	f	18.6	0.4	143	50.0

[a]Shown in Figure 5.

The micro-method was used to re-isolate lipoproteins following incubation. VLDL were separated from LDL or HDL at d = 1.006 g/mL, while LDL was separated from HDL at d = 1.063. The lipoprotein fractions labeled with deuterated tocopherols were frozen and stored at −70°C until shipped by overnight freight on dry ice to the National Research Council in Ottawa, Canada, for analyses of the tocopherols. The radioactively labeled samples were counted as described below.

In some experiments, following incubation (described below) to rapidly re-isolate apolipoprotein (apo) B-containing lipoproteins from HDL, a manganese-heparin precipitation technique was used (17). Briefly, a stock solution was added to each tube such that the final concentrations were: heparin (14 mg/mL) and manganese chloride (0.092 M). The samples were mixed, allowed to stand at room temperature for 10 min, then the tubes were centrifuged for 1 min at 10,000 × *g* and the supernatant (HDL fraction) was transferred to scintillation vials for counting in ACS (Aqueous Counting Scintillant, Amersham, Arlington Heights, IL) using a Beckman LS 7000 scintillation counter. To dissolve the precipitate, 100 μL of 1 M Na citrate was added to each tube and the solution was transferred to scintillation vials for counting, as above.

Incubation protocol. Various combinations of labeled and unlabeled lipoproteins based on protein ratios (as described in the figure legends) were incubated together in a uniform volume (<2 mL) in sterile, plastic tubes in a 37°C water bath with gentle agitation for 2 h, or the indicated interval. The amount of labeled lipoprotein was kept constant, while varying amounts of the unlabeled lipoprotein were added to achieve the indicated protein ratios, then saline (0.15 M NaCl, 0.3 mM EDTA, pH 7.4) was added such that the total volume for the series of protein levels was constant. No other proteins or plasma components were added. Following the incubation, the lipoproteins were re-isolated using the micro-isolation method or the precipitation technique as described in the legends.

The % tocopherol transferred was calculated from the amount of labeled tocopherol (either ^3H or ^2H) in the acceptor lipoprotein fraction divided by the total labeled tocopherol recovered in the donor and acceptor fractions.

RESULTS

Shown in Figure 1 are the time courses (up to 24 h) of the [^{3}H]tocopherol distribution into HDL from labeled LDL, or the reverse, at the indicated donor to acceptor protein ratios. When labeled LDL was incubated with HDL up to 24 h, about 80% of label remained in LDL. By contrast, when labeled HDL was incubated with LDL up to 24 h, about 75% of the label had transferred to LDL. Most of this transfer occurred prior to 30-min. Previously, Massey (6) demonstrated that equilibration of labeled tocopherol from HDL to LDL took place in less than 1 h. Since most of the transfer occurred within the first 2 h, the subsequent incubations were carried out for 2 h.

Shown in Figure 2 are the results of a representative experiment of three in which [^{3}H]tocopherol-labeled donor lipoproteins were incubated with acceptor lipoproteins at protein ratios from 0.33 to 3. Little [^{3}H]tocopherol transferred from labeled VLDL to either LDL or HDL, irrespective of the amount of acceptor (Fig. 2A and C). Similarly, when labeled LDL was the donor at a donor-to-acceptor ratio of 0.5, 17% of the [^{3}H]tocopherol transferred to HDL, while at a ratio of 3, only 7% was transferred (Fig. 2D). Unlike LDL and VLDL, HDL readily donated tocopherol. When labeled HDL was incubated with LDL at a donor-to-acceptor ratio of 0.5 (shown as LDL/labeled HDL = 2, Fig. 2B), 78% of the [^{3}H]tocopherol was transferred to LDL, while at a donor-to-acceptor ratio of 3 (shown as LDL/labeled HDL = 0.3, Fig. 2B), 56% was transferred.

Although this experimental protocol was carried out several times with similar results, it seemed possible that a systematic error was taking place. To isolate the apo B-containing lipoproteins from HDL, a manganese-heparin precipitation technique was used. Since the lipoproteins were labeled *in vitro*, it seemed possible that this procedure might have denatured the lipoproteins and these denatured labeled lipoproteins were precipitated by the reagent and thus were artificially increasing the apparent transfer from HDL to apo B-containing lipoproteins, and were increasing the amount of label retained by apo B-containing lipoproteins, when incubated with HDL. Therefore, in the studies described below metabolically labeled lipoproteins were used—deuterated tocopherols were administered orally to subjects, then plasma was obtained 12–16 h later, and the lipoproteins were isolated before and after incubation using ultracentrifugation techniques.

Figure 3 shows the data from one of three separate experiments in which different donors provided plasma for the incubations; the three experiments yielded similar results. The subject was given both *RRR-* and *SRR-α*-tocopherols labeled with different amounts of deuterium, but only the d_6-*RRR-α*-tocopherol results are shown because the relative transfers of *SSR-* were identical to those of *RRR-α*-tocopherol. Thus, transfer between lipoproteins during 2 h *in vitro* incubations does not result in discrimination between stereoisomers of *α*-tocopherol.

When VLDL was used as a donor of deuterated tocopherols (Fig. 3A and C), only 6–12% was detected in LDL or HDL after a 2-h incubation. LDL was a somewhat better donor of the d_6-tocopherol (Fig. 3 D and F). At a donor-to-acceptor protein ratio of 0.25, 32% of the d_6-tocopherol transferred to HDL, and 27% transferred to VLDL. When the amount of acceptor was decreased to a donor/acceptor ratio of 2, these percentages decreased to 14% and 11%, respectively. By contrast, HDL functioned well as a donor of d_6-tocopherol. At a donor-to-acceptor protein ratio of 0.25, ~90% of the d_6-tocopherol was transferred to VLDL (Fig. 3B), and 87% to LDL (ratio LDL/labeled HDL = 2, Fig. 3E). However, when the amount of acceptor was decreased, less d_6-tocopherol was transferred. For example, at a donor-to-acceptor ratio of 2, 59% was transferred to VLDL (Fig. 3B) and 40% to LDL (LDL/labeled HDL = 0.5, Fig. 3E).

The labeled and unlabeled tocopherol contents of the lipoproteins at the end of the incubation period (from the experiment shown in Fig. 3) are shown in Figure 4. In the incubation of labeled HDL with VLDL (Fig. 4A), the

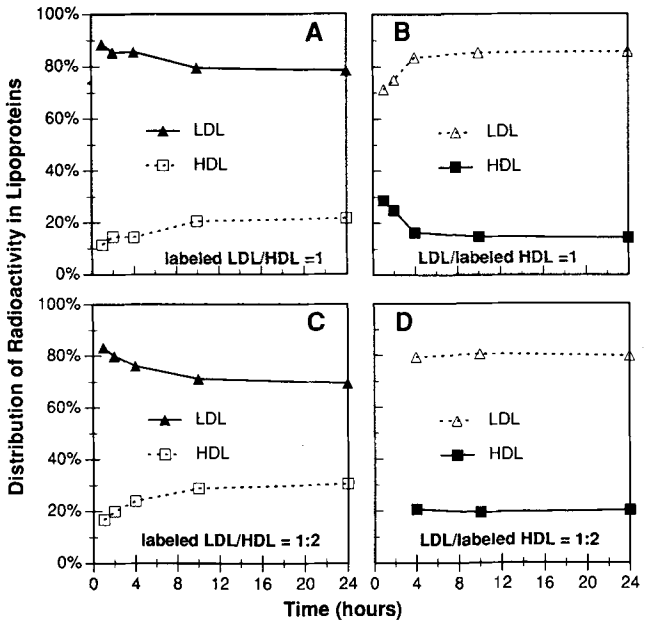

FIG. 1. Time course of the net transfer of [^{3}H]tocopherol between LDL and HDL. Plasma was labeled with [^{3}H]tocopherol and then lipoproteins were isolated by manganese-heparin precipitation as described in Materials and Methods. A constant amount (100 μL) of labeled LDL (4.2 μg protein/μL, 17 ng α-tocopherol/μL, 62.1 cpm/ng tocopherol) was mixed with unlabeled HDL (15.7 μg protein/μL, 17.4 mg tocopherol/μL) at labeled LDL/HDL protein ratios of 1 and 0.5 and volumes were adjusted to 1 mL with saline. Similarly, a constant amount (100 μL) of labeled HDL (5.2 μg protein/μL, 10.9 ng α-tocopherol/μL, 102 cpm/ng α-tocopherol) was mixed with unlabeled LDL (3.26 μg protein/μL, 11.3 ng tocopherol/μL) at labeled HDL/LDL protein ratios of 1 and 2 and volumes were adjusted to 1 mL. Mixtures were incubated in the dark in a 37°C water bath with constant gentle mixing, then 0.2 mL aliquots were taken at the indicated intervals. Immediately, LDL was precipitated from each aliquot by the addition of manganese-heparin (14 mg heparin/mL, 0.092 M Mn), the samples centrifuged, and the HDL supernatants removed and counted. LDL precipitates were resuspended in 1M Na citrate and counted. Shown are the % [^{3}H]tocopherol transferred calculated as the cpm in the acceptor lipoprotein as a fraction of the total cpm per time point.

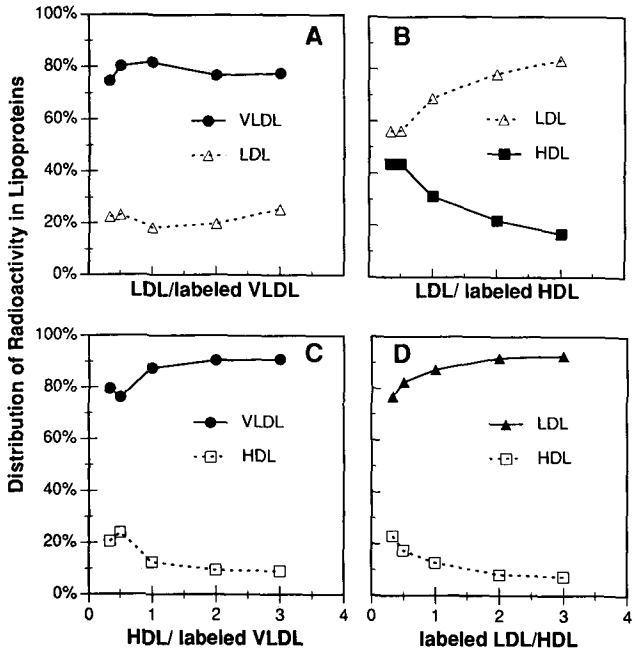

FIG. 2. Transfer of [^{3}H]tocopherol between lipoproteins at various protein ratios. Lipoproteins were labeled and isolated by manganese-heparin precipitation as described in Materials and Methods. A constant amount (260 μL) of labeled LDL (2.0 μg-protein/μL, 10.8 ng α-tocopherol/μL, 12.2 cpm/ng α-tocopherol) was mixed with unlabeled HDL (15.7 μg protein/μL, 17.4 ng α-tocopherol/μL) at labeled LDL/HDL protein ratios of 0.33, 0.5, 1, 2 and 3 and volumes adjusted to 1 mL with saline. Similarly, a constant amount (260 μL) of labeled HDL (6.6 μg protein/μL, 10.1 ng α-tocopherol/μL, 8.4 cpm/ng α-tocopherol) was mixed with unlabeled LDL (3.26 μg protein/μL, 11.3 ng tocopherol/mL) at the same protein ratios and volumes. For the transfer from VLDL to HDL (labeled VLDL -> HDL) two similar experiments are shown, but with different amounts of tocopherol in the donor VLDL. In the first experiment a constant amount (130 μL) of labeled VLDL (1.13 μg protein/μL, 7.9 ng α-tocopherol/μL, 50.7 cpm/ng α-tocopherol) was mixed with either unlabeled LDL or HDL (protein contents as above) at the same protein ratios (labeled VLDL/HDL or labeled VLDL/LDL) as above. In the second experiment (shown as a dotted line) a constant amount (85 μL) of labeled VLDL (2.39 μL, 9.6 ng α-tocopherol/μL, 19.6 cpm/ng α-tocopherol) was mixed with the same unlabeled HDL as above. Mixtures were incubated for 2 h as in Figure 1. After incubation, LDL and VLDL were separated from HDL by precipitation with Mn-heparin, VLDL was separated from LDL by the micro-method of ultracentrifugation described in the Methods section, and lipoprotein fractions were counted and the % [^{3}H]tocopherol transferred was calculated as described in Figure 1.

large amount of unlabeled tocopherol in the acceptor VLDL might be the reason for the nearly complete transfer of d_6-tocopherol from HDL to VLDL. However, the range of levels of unlabeled tocopherol in VLDL in this incubation was similar to that in the labeled LDL incubation with VLDL, shown in Figure 4D. The fact that a majority of d_6-tocopherol remained in LDL, despite the large quantities of unlabeled tocopherol in VLDL, suggests that the structure and composition of the donor lipoproteins (HDL *vs.* LDL) is more important than the content of unlabeled tocopherol in the acceptor lipoproteins. This is further amplified in Figure 4F showing the incubation of labeled VLDL with LDL at a protein ratio of 0.5. Here the d_0-tocopherol concentrations in LDL and VLDL are equal, yet the d_6-tocopherol remains in VLDL.

It is also interesting to compare the transfers between LDL and HDL. As shown in Figure 4C, when labeled LDL was incubated with HDL, the d_0-tocopherol content varied from double in the acceptor HDL to less than 10% of the LDL tocopherol, but this had relatively little effect on the transfer of d_6-tocopherol to HDL with most being retained in LDL; a small amount of d_6-tocopherol increased in HDL with increasing d_0-tocopherol in the acceptor HDL. By contrast, the transfer from labeled HDL to LDL (or VLDL) did closely parallel the amount of unlabeled tocopherol in the acceptor (Fig. 4B and A, respectively).

The results of these studies suggested that the amount of tocopherol in HDL *in vivo* might be dependent upon the ratio of circulating LDL to HDL in the plasma. That is, the higher the LDL concentration in the plasma, the greater the transfer of tocopherol to LDL and thus the lower the tocopherol per HDL particle, and conversely in subjects with a high proportion of HDL to LDL, the tocopherol per HDL particle might be increased. Therefore, the lipoprotein distribution of plasma tocopherol in 11 subjects undergoing routine evaluations of their lipid levels was also carried out. The characteristics of these subjects are shown in Table 1. To be noted is the wide range of HDL values. The fraction of plasma cholesterol in HDL (% HDL) ranged from 14 to 50%. Shown in Figure 5 are the tocopherol/protein and the cholesterol/protein ratios in HDL as a function of the % HDL cholesterol in the plasma. There was a significant correlation (r^2 = 0.366, $P < 0.05$) between the ng tocopherol/mg HDL protein and the % HDL cholesterol, while the relationship between the cholesterol/protein ratio and % HDL cholesterol was not correlated. Furthermore, neither the tocopherol/protein ratio, nor the cholesterol/protein ratio in LDL was correlated with the % LDL cholesterol in these subjects (data not shown).

DISCUSSION

We demonstrate that within a 2-h incubation, apo B-containing lipoproteins, both LDL and VLDL, retain a majority of the labeled tocopherol and do not readily donate it to other lipoproteins. Less than 40% of the labeled tocopherol was found in acceptor lipoproteins even at high-acceptor-to-donor ratios (Fig. 2 and 3). By contrast, HDL was found to readily donate labeled tocopherol either to LDL or to VLDL. Patients with abetalipoproteinemia are lacking apolipoprotein-B containing lipoproteins and transport lipids solely in HDL. These patients seldom have vitamin E levels more than one-tenth of control values. Thus, it is likely that HDL readily transfers tocopherol to tissues in these patients.

The ability of HDL to donate tocopherol was demonstrated using two different means of labeling the lipoproteins with tocopherol, both an *in vitro* technique using [^{3}H]tocopherol and *in vivo* metabolic labeling using deuterated tocopherols. Furthermore, two isolation techniques were used to isolate the lipoproteins following incubation. In the precipitation technique, the apo B-containing lipoproteins are precipitated, while in the ultracentrifugation technique the apo B lipoproteins are floated to the top of the tube. Since the two different methodologies yielded similar results, the conclusion that HDL readily donates tocopherol to LDL or VLDL is independent of

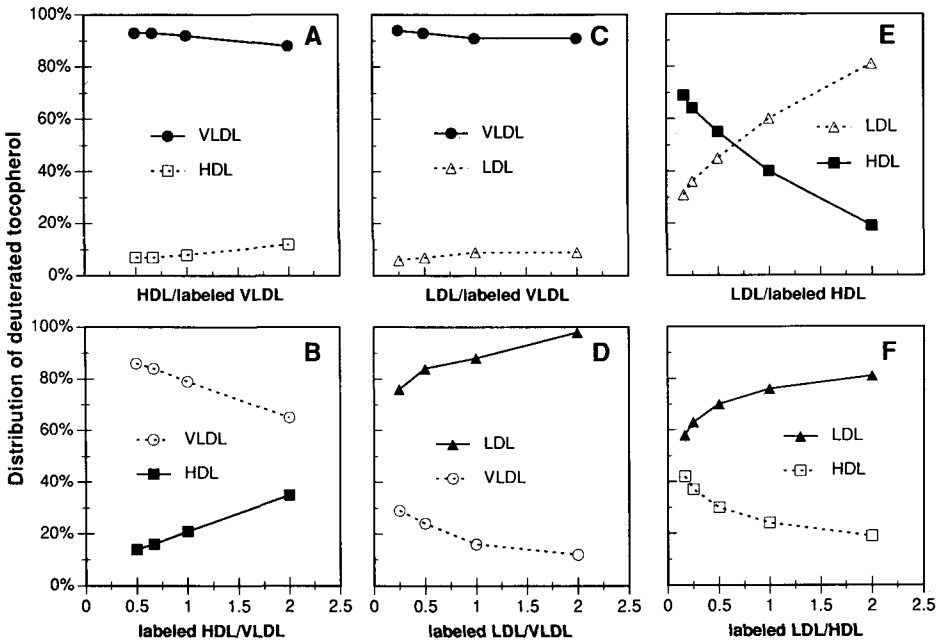

FIG. 3. Transfer of deuterium-labeled tocopherol between lipoproteins. Lipoproteins were isolated by ultracentrifugation from the plasma of a normal human subject who ingested 100 mg each of d_6-*RRR*-alpha-tocopheryl acetate (2*R*,4′*R*,8′*R*-α-[5,7-(C^2H_3)$_2$]tocopheryl acetate) and d_3-*SRR*-α-tocopheryl acetate (2*S*,4′*R*,8′*R*-α-]5-C^2H_3]tocopheryl acetate) as described in Materials and Methods. The Figure is representative of three experiments with similar results; only d_6-*RRR* values are shown, as d_6-*RRR* and d_3-*SRR*-α-tocopherol transfer values were similar. The protein (pro) and tocopherol contents of the starting lipoproteins per mL were: labeled VLDL 1.1 mg pro, 24.2 nmol d_0 and 8.6 nmol d_6; labeled LDL 1.1 mg pro, 20.7 nmol d_0 and 6.1 nmol d_6; labeled HDL 10.5 mg pro, 38.9 nmol d_0 11.34 nmol d_6; unlabeled VLDL 1.3 mg pro and 79.0 nmol d_0; LDL 2.7 mg pro and 33.6 nmol d_0; and HDL 7.3 mg pro and 13.7 nmol d_0. Labeled HDL (100 μL) was mixed with unlabeled VLDL at labeled HDL/VLDL protein ratios of 0.5, 0.67, 1 and 2, then taken to a volume of 1.7 mL; and with unlabeled LDL at labeled HDL/LDL protein ratios of 0.5, 1, 2, 4, and 6, then taken to a volume of 0.85 mL. Labeled VLDL (400 μL) was mixed with unlabeled HDL at labeled VLDL/HDL protein ratios of 0.5, 1, 1.5 and 2, then taken to a volume of 0.525 mL; and with unlabeled LDL at labeled VLDL/LDL protein ratios of 0.5, 1, 2 and 4, then taken to a volume of 0.725 mL. Labeled LDL (250 μL) was mixed with unlabeled VLDL at labeled LDL/VLDL protein ratios of 0.25, 0.5, 1 and 2, then taken to a volume of 2 mL; or labeled LDL (300 μL) was mixed with unlabeled HDL at labeled LDL/HDL protein ratios of 0.167, 0.25, 0.5, 1 and 2, then taken to a volume of 0.8 mL. All incubations were performed at 37°C in the dark for 2 h. At the end of the incubation period, the lipoproteins were immediately isolated by the micro-ultracentrifugation technique, and tocopherols in each fraction were measured as described in Materials and Methods. The % distribution of the d_6-tocopherol is calculated as the d_6-tocopherol in each lipoprotein as a fraction of the total d_6-tocopherol recovered at each protein concentration.

the isolation technique used. Furthermore, in data not shown, use of the precipitation technique following incubation of deuterated tocopherols also led to the same conclusions.

It is interesting that the stereochemistry of the 2-position did not affect the transfer of tocopherol between lipoproteins. As noted, but not shown, both *RRR* and *SRR*-α-tocopherol exchanged in an identical manner between the lipoproteins, suggesting that the structural differences between these two α-tocopherols were not sufficient to alter exchange rates. However, in one preliminary experiment we studied the exchange of deuterated cholesterol and d_6-*RRR*-α-tocopherol lipoproteins with unlabeled lipoproteins using an identical protocol to that described in Figure 3. When labeled LDL was incubated with HDL, although 80% of the labeled tocopherol remained in LDL, the labeled cholesterol equilibrated with about 50% in each fraction. These data suggest that the equilibration of tocopherol between lipoproteins is not dependent generally upon lipid flux, but is specific for each lipid studied, and is dependent upon the lipid and protein composition of the donor and acceptor lipoproteins. This is consistent with the studies of Granot *et al.* (9), who found that transfer of vitamin E remained relatively constant (40–55%) while triglyceride transfer ranged from 20 to 80%. Lund-Katz *et al.* (18) in their studies of cholesterol exchange between HDL and LDL concluded that their kinetic data was consistent with desorption of cholesterol from the donor lipoprotein into the aqueous phase, then cholesterol diffusing through the aqueous phase to the acceptor. The exchange of cholesterol from the core of HDL to the surface was not rate limiting for this exchange. From our data and the known characteristics of lipoproteins (19), we can hypothesize as to why tocopherol is more likely to be transferrd by HDL than the apo B-containing lipoproteins. A tocopherol molecule on the surface of HDL is perhaps more likely to desorb into the aqueous-phase than into the core, because although the HDL core is cholesteryl ester-rich (81 mol% *vs.* 19 mol % triglycerides), HDL is a relatively small lipoprotein (175,000–360,000 MV) with about 50% of the weight as protein, and 2–5% of the surface

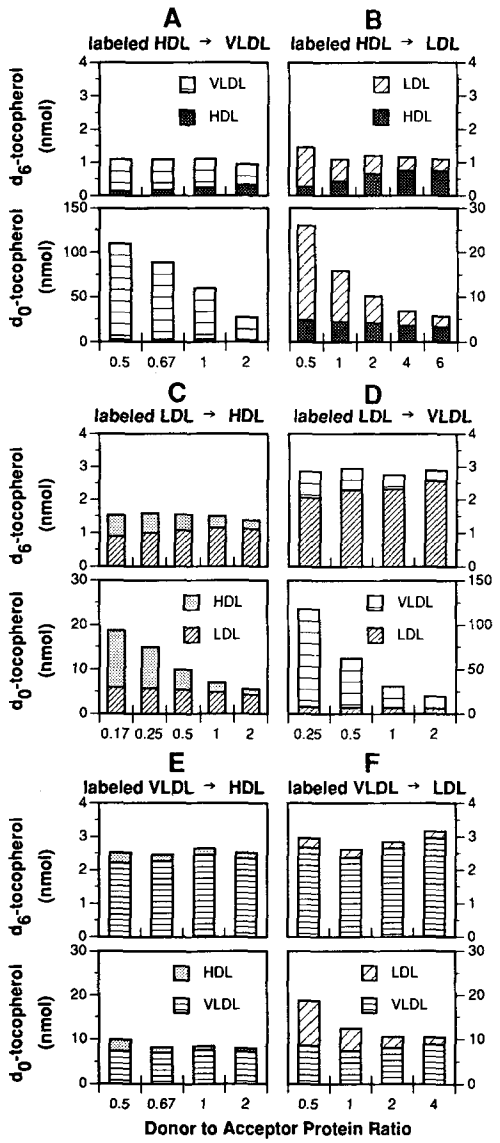

FIG. 4. Tocopherol contents of lipoprotein fractions from Figure 3. Each pair of graphs depicts the absolute amounts of d_6- and d_0-α-tocopherol (nmol) measured in each lipoprotein fraction after incubation and isolation, shown in Figure 3. Donor lipoproteins are shown with a denser pattern.

components are protein (on a mol% basis) LDL, which is 10 times larger than HDL 2,300,000 MW), has less protein on the surface (0.2%) but has the same distribution of core lipids. Thus, tocopherol is less likely to desorb into the aqueous phase, as the larger lipid core in LDL acts as a sink for the tocopherol. A tocopherol molecule on the surface of VLDL (10,000,000–80,000,000 MW) is most likely to dissolve in the core, which is 95 mol% triglyceride, than to desorb into the aqueous phase. Thus, we suggest that a tocopherol molecule moves readily between surface and core, and within the core, but not between the surface and the aqueous phase. By similar mechanisms, if either LDL or VLDL functions as the acceptor, then it is likely that there will be little transfer back to the donor lipoproteins, but if HDL acts as a tocopherol acceptor, the tocopherol will readily transfer back to the donor. Thus,

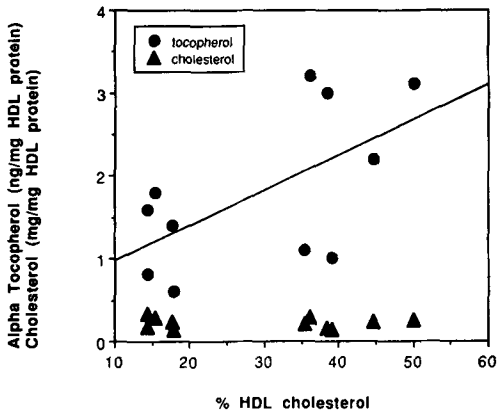

FIG. 5. Tocopherol and cholesterol concentrations per HDL protein, as a function of % HDL cholesterol in the plasma of human subjects. Following an overnight fast, blood samples were collected from 11 human subjects. The tocopherol content of HDL based on the nmol/mg HDL protein was significantly correlated with the proportion of HDL expressed as a % of total plasma cholesterol (r^2 = 0.366, $P < 0.05$), while the cholesterol content was not (r^2 = 0.039).

the net transfer of tocopherol from HDL will be enhanced by the larger lipid core of VLDL compared to LDL, while the transfer from VLDL will not be influenced by the acceptor because little tocopherol will escape the VLDL (as shown in Fig. 3).

Based on the observation that the HDL tocopherol was dependent upon the ratio of LDL/HDL, we investigated the tocopherol content of lipoproteins isolated from subjects with LDL/HDL protein ratios varying from 0.3 to 1.6. We found that as the fraction of HDL cholesterol increased in the plasma the amount of tocopherol per protein in HDL also increased. Although only 11 subjects were investigated, this was a statistically significant phenomenon. Thus, a person who has a high HDL level is likely to retain more tocopherol in their HDL fraction, than those with a high LDL. Both Behrens and Madere (20) and Clevidence and Lehmann (21) have reported that HDL α-tocopherol is related to HDL protein concentrations. The physiological importance of this observation needs further investigation. These *in vitro* studies also have relevance to the *in vivo* studies of tocopherol transport in lipoproteins. It is likely that transfer of tocopherol to HDL occurs *in vivo* during the catabolism of chylomicrons, because there is transfer of surface and core components between chylomicrons and HDL during the action of lipoprotein lipase. From the *in vitro* incubations described in the present study, it is clear that the tocopherol in HDL is readily transferred to both VLDL and LDL. At the highest labeled HDL/VLDL protein ratio tested (2:1), 65% of the d_6-tocopherol in HDL was transferred to VLDL, with greater proportions at higher VLDL concentrations (Fig. 3B). Thus, VLDL, and perhaps chylomicron remnants, present in the circulation during chylomicron catabolism, are likely to serve as tocopherol acceptors, returning plasma tocopherol to the liver during triglyceride-rich lipoprotein uptake.

In conclusion, this study has demonstrated that tocopherol in HDL readily transfers to apo B-containing lipoproteins, with little return of tocopherol from the

apolipoprotein B-containing lipoproteins to HDL. The transfer of tocopherol from HDL to LDL appears to depend upon the ratio of the lipoproteins present; with tocopherol transferring to LDL at LDL/HDL ratios of one or greater. An increased proportion of tocopherol/protein was found in HDL isolated from subjects with a high proportion of HDL in their plasma.

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Lipoprotein Receptor Mediated Metabolism of [^{14}C]Arachidonic Acid Labeled Chylomicron Remnants by Hep G2 Cells

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During lipolysis of chylomicron triacylglycerol by lipoprotein lipase, arachidonic acid (AA) esters are hydrolyzed at a slower rate than the predominant 16–18 carbon fatty acid esters. The further metabolism of the AA that is hereby enriched in the chylomicron remnant acylglycerols has not been investigated. In the present study, we examined the low density lipoprotein (LDL) dependent and independent metabolism of [^{14}C]AA present in chylomicron remnants in the human hepatoma cell line Hep G2. Mesenteric duct cannulated rats were fed [^{14}C]AA and [^{3}H]cholesterol in corn oil, and the chyle obtained was injected intravenously into hepatectomized rats to form chylomicron remnants labeled with [^{14}C]AA in the triacylglycerol (TG) and with ^{3}H in the cholesteryl ester portion. The remnants were then incubated with Hep G2 cells. The uptake of [^{14}C]AA within 2–4 h was similar to that of [^{3}H]cholesteryl ester. After uptake into the cells, [^{14}C]AA was preferentially incorporated into phospholipids, a high proportion being found in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. [^{14}C]AA and [^{3}H]cholesteryl ester uptake were influenced to similar extents by factors unknown to regulate the LDL receptor and by an anti-LDL receptor antibody. Addition of compactin thus increased the uptake of [^{14}C]AA by 50% in 4 h and mevalonolactone decreased the uptake by 86%. Using an anti-LDL receptor antibody, 25.0% of [^{3}H]cholesterol/cholesteryl ester and 37.7% of [^{14}C]AA binding to the cells at 4°C were blocked. There was no lipolysis of [^{14}C]TG or [^{14}C]diacylglycerol by lipase secreted into the medium during incubations. The study shows that after the uptake of chylomicron remnants by Hep G2 cells, which in part occurs *via* the LDL receptor, AA is liberated from the acylglycerols and is preferentially incorporated into phospholipids.

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Significant amounts of arachidonic acid (AA) are transported by chyle lipoproteins. Most of this AA must originate from bile phosphatidylcholine (PC) which, in the rat, contains about 20% AA at the *sn*-2 position (1). In thoracic duct cannulated rats, the output of AA in chyle was 1.2 μmol/h during fasting, and 2.4 and 1.9 μmol/h after feeding moderate doses of Intralipid® or cream (2). A considerable part of this AA was in chyle phospholipids (2) which may be metabolized by lipoprotein lipase (LPL) (3,4) or transferred to high density lipoproteins (HDL) (5) and metabolized by hepatic lipase (6,7) or lecithin:cholesterol acyltransferase (LCAT) (8,9). A major part of the AA was, however, still incorporated into the chyle triacylglycerol (TG) (2).

During lipolysis of chylomicron TG by LPL, the AA esters exhibited a relative resistance to LPL (10). AA was therefore enriched in TG and in 2,3-diacylglycerol (DG) of the chylomicron remnants formed, the hydrolysis being somewhat more efficient when hepatic lipase was combined with LPL during the incubation (10). The metabolism of the remnant particles may thus have a more important role in the tissue distribution of AA than in that of the predominant dietary 16–18 carbon fatty acids. Chylomicron remnants are rapidly cleared and catabolized in hepatocytes (11) by an endocytotic process (12) that is mediated by apolipoprotein E and modulated by apolipoprotein C and phospholipids (13–18). The role of the LDL receptor in the remnant clearance is uncertain. Remnants are taken up *via* the low density lipoprotein (LDL) receptor in cultured hepatocytes (19), but both a normal (20) and a delayed remnant clearance (21,22) has been reported in Watanabe heritable hyperlipidemic rabbits (WHHL), which lack the LDL receptor. Another pathway which involves binding of apolipoprotein E of the remnant particles may be the predominant pathway *in vivo* (23). The uptake by this pathway might depend on the LDL receptor related protein (LRP), which has thus been considered a hypothetical remnant receptor (24,25). LRP recently was found to be identical to the alpha 2 macroglobulin receptor (26). In this study the metabolism of [^{14}C]AA present in chylomicron remnant acylglycerols was examined in the human hepatoblastoma cell line Hep G2. This cell line expresses LDL receptors (27) as well as LRP, and also secretes the enzyme hepatic lipase (28,29). The purpose was to obtain further information about the pathways by which the remnant AA is taken up and further metabolized by liver cells.

MATERIALS AND METHODS

Cell culture. The hepatoblastoma cell line Hep G2 was obtained from American Tissue Culture Collection (Rockville, MD). Cells were cultured at 37°C in 75-cm^2 flasks in RPMI 1640 (Gibco Laboratories, Santa Clara, CA) containing 10% fetal calf serum (heat inactivated at 56°C for 30 min, from Flow Laboratories Irvine, Ayrshire, Scotland), in the presence of benzyl penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were grown in humidified air with 5% CO_2 and were subcultured approximately once a week at a split ratio of 1:6.

Cells were trypsinized and then seeded into plastic Petri dishes. The medium (RPMI 1640 with 10% fetal calf serum without antibiotics) was routinely changed after two days. Preincubations with different additions indicated were started after a week. During the incubation with remnants, cells were grown in serum-free medium with 0.5% human serum albumin. The cells were routinely screened for mycoplasma using a kit with a specific

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Abbreviations: AA, arachidonic acid; DG, diacylglycerol; EDTA, ethylenediaminetetraacetate; FCS, fetal calf serum; FFA, free fatty acids; HDL, high density lipoprotein; HMG-CoA, hydroxy methyl glutaryl coenzyme A; LCAT, lecithin: cholesterol acyltransferase; LDL, low density lipoprotein; LPL, lipoprotein lipase; LRP, LDL receptor related protein; PBS, phosphate buffered saline; PC, phosphatidylcholine; PDGF, platelet-derived growth factor; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; TG, triacylglycerol; WHHL, Watanabe heritable hyperlipidemic rabbits.

DNA probe from Gen-Probe Inc. (San Diego, CA). No mycoplasma was found.

Preparation of labeled chylomicron remnants. Mesenteric lymph duct cannulations were performed according to Warshaw (30) and a feeding fistula was inserted with the tip placed in the proximal duodenum. The rats were treated postoperatively, as described earlier (31). They were fed 1.0 mL corn oil containing 20 mg unlabeled cholesterol, 500 μCi (1.85 MBq) [1,2-^{3}H]cholesterol and 10 μCi [1-^{14}C]AA (51.3 mCi/mmol). Radioactive cholesterol and radioactive AA were obtained from Amersham International (Amersham, U.K.) and New England Nuclear (Boston, MA). The fat diets were divided into three doses and given through the feeding fistula over 2 h. Lymph was collected and stored at 4°C in the presence of 2 mM Na_2-EDTA (ethylenediaminetetraacetate; ref. 32). Chylomicron remnants were prepared by injecting chyle into eviscerated rats (33). After 30 min, blood was drawn from the abdominal aorta using Na_2-EDTA as anticoagulant. The density of the plasma was adjusted to 1.063 g/mL by adding a stock solution (d = 1.35 g/mL, 354 g/L KBr and 153 g/L NaCl). Plasma was then layered under saline (d = 1.006 g/mL) and centrifuged in a Beckman L5-65 ultracentrifuge (Fullerton, CA) with a SW Ti 40 rotor at 32,000 rpm (120,000 × *g*) for 12 h. The top layer containing chylomicron remnants was collected under sterile conditions and used within 24 h. The particles prepared by injecting chyle into eviscerated rats had lost 67.5% of their original TG content, as estimated from the change in the [^{14}C]TG/[^{3}H]cholesteryl ester ratio in chylomicron remnants in comparison to that of the injected chyle. The proportion of [^{3}H]cholesterol in cholesteryl ester of chylomicron remnants was 75.5%. The specific activity of the radioactively labeled cholesterol ranged between 5.3–6.4 dpm ^{3}H/ng cholesterol.

Incubation of chylomicron remnants with Hep G2 cells. The labeled chylomicron remnants were incubated with Hep G2 cells either at 37°C for up to 4 h or at 4°C for 2 h. At 37°C both the uptake and net degradation of chylomicron remnant cholesteryl esters by the cells were measured. At 4°C only binding of chylomicron remnants to cells was measured. After the incubation period the cells were washed twice with 2 mL ice cold phosphate buffered saline (PBS) containing 0.2% bovine serum albumin and then twice with 2 mL PBS without albumin. The cells were scraped off the dishes with a rubber policeman during repeated additions (2 × 1 mL) of methanol/water (2:1, vol/vol). Lipids were extracted from cells and media with chloroform/methanol (1:2, vol/vol) (34). Nonpolar lipids were separated by thin-layer chromatography on silica gel G plates, which were developed in light petroleum/diethyl ether/acetic acid (80:20:1, vol/vol/vol). Plates were developed in chloroform/methanol/acetic acid/water (100:80:12:1.2, by vol) to separate phospholipids. Spots were made visible by staining with iodine vapor, and scraped into vials. Methanol/water (1 mL; 1:1, vol/vol) and Instagel/toluene (9 mL; 1:1, vol/vol) were added, and radioactivity was determined in a Packard TC 460 CD Liquid Scintillation System (Packard Instruments, Downers Grove, IL) using the computerized external standard for quench correction.

Net hydrolysis of chylomicron remnant cholesteryl esters was calculated as described earlier (31). The percentage net hydrolysis of radioactive cholesteryl ester was calculated from the decrease in radioactivity (dpm) of cholesteryl ester, and the increase in that of free cholesterol in both cells and medium as follows:

$$\% \text{ net hydrolysis} = 100 - \frac{\% \text{ cholesterol as ester after incubation} \times 100}{\% \text{ cholesterol as ester before incubation}}$$

The percentage net hydrolysis was then multiplied by the dpm cholesteryl ester added to the dish to obtain dpm cholesteryl ester hydrolyzed. No measurable net hydrolysis of lipoprotein cholesteryl ester was observed in the cell-free controls. The uptake of chylomicron remnant cholesteryl ester (Fig. 1) is the amount of cholesteryl ester found in the washed cells as a percentage of added ^{3}H to the medium plus the percentage of net hydrolysis of [^{3}H]cholesteryl ester. The percentage of AA uptake in Figure 1 and Table 1 was calculated as follows:

$$\% \text{ AA uptake} = \frac{^{14}\text{C dpm in cells after incubation}}{^{14}\text{C dpm in remnants before incubation}} \times 100$$

LDL receptor antibody. The monoclonal anti-LDL receptor antibody (clone C7) was obtained from Amersham (Buckinghamshire, England). This antibody blocks LDL receptor mediated uptake of LDL in cultured human fibroblasts (35). In earlier experiments we have shown that approximately 50% of the LDL binding to Hep G2 cells is blocked (36).

Chemical assays. Triacylglycerol and cholesterol were determined using the commercial enzymatic kits from Boehringer Mannheim (Mannheim, Germany). The protein contents of the cells and of LDL were determined by the Lowry method (37) using human serum albumin as a standard.

RESULTS

When chylomicron remnants were incubated with Hep G2 cells, uptake of ^{3}H-labeled cholesteryl ester, ^{3}H-labeled cholesterol and ^{14}C-labeled AA occurred. The time courses for the uptakes of [^{14}C]AA and of [^{3}H]cholesteryl ester were similar (Fig. 1). The uptake of AA as a percentage of added label to the medium differs from that of cholesteryl ester during the first 1 h incubation ($P < 0.05$). The uptake of [^{14}C]AA and of [^{3}H]cholesteryl ester after 2 and 4 h did not differ.

The effects of factors which are known to influence cellular cholesterol content and LDL receptor levels on the uptake of chylomicron remnants were examined. When cells were incubated for 24 h with LDL to increase cellular cholesterol contents, AA uptake decreased to 3.8%, compared to 5.7% in controls (Table 1). When cellular cholesterol synthesis was stimulated by adding mevalonolactone, AA uptake decreased even more (Fig. 2). After incubation of the cells with lipoprotein-deficient medium (Table 1) or with compactin (Fig. 2), AA uptake increased by approximately 50%. The variation of AA uptake under these conditions closely followed that of cholesteryl ester

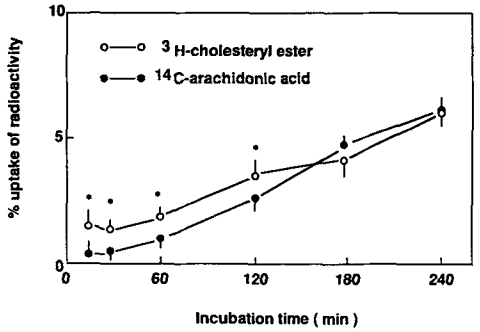

FIG. 1. Time course of uptake of [^{14}C]AA and [^{3}H]cholesteryl ester by Hep G2 cells. Chylomicron remnants (50 μL, 2.7 μg of cholesteryl ester and 28.5 μg of TG), 1.7-2.1 × 10,000 dpm of ^{3}H and 1.3-5.4 × 1,000 dpm of ^{14}C were added to each dish of Hep G2 cells and incubated for various time intervals. After the incubation, cells and medium were treated as described in Materials and Methods. Each time point represents the mean of five dishes. $*P < 0.05$.

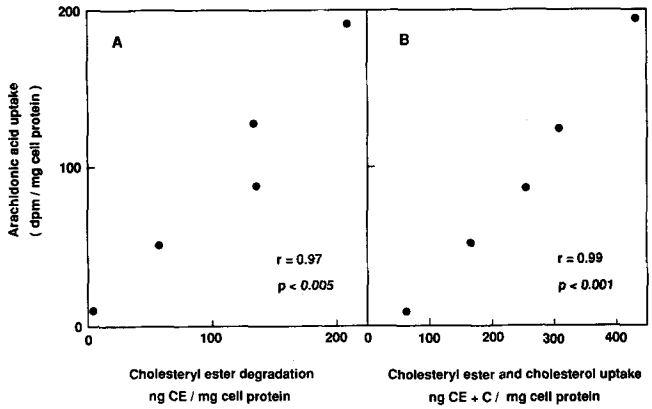

FIG. 3. Correlations between cholesteryl ester degradation and AA uptake (A) and cholesteryl ester and cholesterol uptake and AA upotake (B) in Hep C2 cells. Cells were preincubated under different conditions for 48 h. As in Figure 2, cells were incubated with 50 μL of chylomicron remnants containing 10 μg cholesteryl ester for 4 h. Each point is the mean of two dishes. Results are from one of similar experiments.

TABLE 1

Uptake of [^{14}C]Arachidonic Acid by Hep G2 Cells[a]

Preincubation conditions	AA uptake	
	dpm/mg Cell protein	% of AA added
1% Fetal calf serum	39.1	5.7
10% Fetal calf serum LDL 200 μg	25.8	3.8
Serum free medium	47.5	6.9
10% Lipoprotein-deficient serum	52.0	7.6

[a]Data are means of five observations. Cells were preincubated with different additions as indicated for 24 h. Then medium was changed to RPMI + 0.5% human serum albumin and incubated with 100 μL chylomicron remnants containing 2.7 μg cholesteryl ester for 4 h.

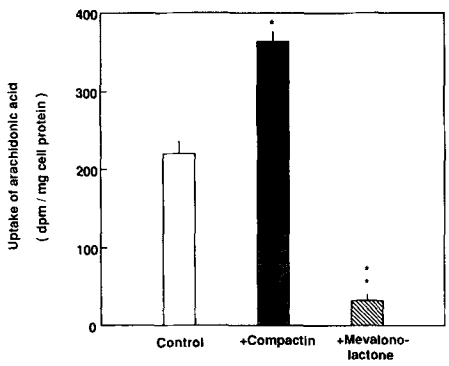

FIG. 2. Effects of compactin and mevalonolactone on uptake of AA in Hep G2 cells. Cells were preincubated in 10 mM Hepes RPMI medium plus 1% fetal calf serum (control) and with medium, FCS and compactin 20 μM (final conc.) or mevalonolactone 10 mM (final conc.). Cells were incubated at 37°C for 4 h with 70 μL chylomicron containing 4.0 μg cholesteryl ester. Uptake of AA was calculated as described in Materials and Methods. $*P < 0.01$, $**P < 0.001$. Results are from one of similar experiments.

degradation (r = 0.97, $P < 0.005$) and cholesterol plus cholesteryl ester uptake (r = 0.99, $P < 0.001$) (Fig. 3).

Addition of an anti-LDL receptor antibody (5 μg/mL) decreased the binding of chylomicron remnants. In eight different experiments 25.0 ± 17.0% (mean ± SE) of ^{3}H-labeled cholesterol plus cholesteryl ester binding to the cells at 4°C was blocked. The corresponding figure for [^{14}C]AA binding was 37.7 ± 23.4%.

After AA had been taken up by the cells, it was reincorporated mainly into the phospholipids. In chylomicron remnants 0.9% of the [^{14}C]AA was found in phospholipids. After incubation with cells, the proportion of ^{14}C found in phospholipids in the medium was increased. After a 4-h incubation, the proportion of cellular ^{14}C radioactivity in phospholipids was 76.0% (Table 2); of this radioactivity, 52.5% was in PC, 31.1% in phosphatidylethanolamine (PE) and 14.6% in phosphatidylinositol (PI) (Table 3). The proportion of the ^{14}C found in TG in the medium after 4-h incubation remained similar to that of the added remnants, and there was only a slight decrease in the proportion found in DG (Table 2). No increase was observed in the proportion of ^{14}C found as free fatty acids in the medium.

DISCUSSION

The present study shows that [^{14}C]AA present in acylglycerols (TG and 2,3-DG) of chylomicron remnants is taken up by Hep G2 cells. The major part of the added remnant [^{14}C]AA was in TG and DG, whereas most of the cellular AA was in phospholipids (Table 2). A net degradation of [^{14}C]acylglycerols and a preferential incorporation of [^{14}C]AA into PC, PE and PI thus occurred (Table 3).

Earlier studies indicate the existence of two types of lipoprotein receptor in liver cells: i) a remnant receptor capable of high-affinity binding of triglyceride-rich lipoproteins and HDL-E, but not of apolipoprotein E-free

TABLE 2

Incorporation of Arachidonic Acid (AA) into Phospholipids (PL): Percentage Distribution of [^{14}C]AA Among Different Lipid Fractions[a]

	PL	1,2-X-DG	Free fatty acids (FFA)	TG
Added remnants	0.9 ± 0.8	11.6 ± 0.5	0.3 ± 0.3	85.7 ± 2.2
Medium				
15 min	2.3 ± 0.8	12.5 ± 0.8	0.2 ± 0.2	82.1 ± 1.0
30 min	2.3 ± 0.8	9.5 ± 0.9	0.1 ± 0.1	83.9 ± 1.5
1 h	1.8 ± 0.4	9.3 ± 1.3	—	84.9 ± 2.0
2 h	2.9 ± 1.2	9.3 ± 1.1	0.1 ± 0.1	84.8 ± 1.1
3 h	2.4 ± 1.3	10.1 ± 1.0	0.1 ± 0.1	82.2 ± 0.8
4 h	2.4 ± 1.1	7.6 ± 1.3	0.2 ± 0.1	85.9 ± 2.6
Cells				
15 min	4.8 ± 2.3	—	—	95.2 ± 4.8
30 min	7.4 ± 3.5	—	—	92.6 ± 7.4
1 h	12.9 ± 3.2	—	—	86.2 ± 7.6
2 h	58.8 ± 1.0[b]	—	—	40.8 ± 0.9[b]
3 h	56.4 ± 7.4[b]	—	—	40.6 ± 5.1[b]
4 h	76.0 ± 4.9[b]	—	—	23.5 ± 4.6[b]

[a]Data are means ± SE of four observations. DG, diacylglycerols; TG, triacylglycerols.
[b]$P < 0.05$, compared to added remnants and medium.

TABLE 3

Distribution of [^{14}C]AA among Phospholipid Classes in Hep G2 Cells[a]

Synthesis of PL	Distribution (%)
Phosphatidic acid	0
Phosphatidylethanolamine	31.1 ± 0.8
Phosphatidylinositol	14.6 ± 0.9
Phosphatidylserine	1.8 ± 0.2
Phosphatidylcholine	52.5 ± 1.3
Lysophosphatidylcholine	0

[a]Data are means ± SE of four observations. Hep G2 cells were incubated with 100 μL chylomicron remnants in RPMI medium + 0.5% human serum albumin for 4 h. AA, arachidonic acid; PL, phospholipid.

LDL, and ii) a LDL receptor capable of high-affinity binding of LDL HDL-E (38) and remnants. *In vivo* the hepatocytes catabolize rat chylomicron remnants by an endocytotic process after the lipoprotein is bound with high affinity to the cells (12). The Hep G2 cells express both LDL receptors and LRP which might be identical with the remnant receptor and with the alpha 2 macroglobulin receptor (26). In an earlier study, the uptake of chylomicron remnant [^{3}H]cholesteryl ester by Hep G2 cells was in part inhibited by an anti-LDL receptor antibody and was influenced by factors altering cell cholesterol contents and, thereby, the LDL-receptor levels, such as mevalonolactone and the hydroxy methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitor compactin (36).

In this study, the time course for the uptake of remnant [^{14}C]AA was similar to that for the uptake of remnant cholesteryl ester, suggesting that the [^{14}C]TG uptake occurred by uptake of the whole remnant particles, possibly by receptor mediated endocytosis (Fig. 1). The percentage of [^{14}C]AA uptake was somewhat lower than that of total [^{3}H] radioactivity, but this may be due to some uptake of unesterified cholesterol by exchange processes. In addition, a certain percentage of AA may be oxidized to CO_2 and H_2O or further metabolized by the cells to prostanoids.

The addition of compactin and mevalonolactone caused significant changes in AA uptake (Fig. 2), which were correlated with changes in uptake of chylomicron remnant cholesteryl ester (Fig. 3B). For example, mevalonolactone inhibited [^{14}C]AA uptake by 86% and [^{3}H] cholesteryl ester uptake by 64%. Also, when the remnant binding of cholesteryl ester and cholesterol was blocked by an anti-LDL receptor antibody, a similar decrease in the uptakes of cholesterol and of AA was observed. The study thus supports the idea that LDL receptors are important for the uptake and degradation of chylomicron remnant [^{14}C]AA-acylglycerols in Hep G2 cells. Because the uptake was only in part inhibited by the anti-LDL antiserum, however, the participation of other pathways must be postulated. Whether the uptake that did not depend on the LDL recepor was mediated by LRP remains to be established.

Hep G2 cells also secrete enzymes participating in lipoprotein metabolism such as hepatic lipase (28,29) and LCAT (39). In this study, the percentage of distribution of [^{14}C]AA in different lipid fractions in the medium remained constant during incubation (Table 2). Thus, there is no evidence for hydrolysis of [^{14}C]AA labeled remnant TG and DG in the medium. The data therefore indicate that the [^{14}C]acylglycerols were hydrolyzed after uptake by the cells, which is consistent with a role of the lipoprotein receptors rather than secreted hepatic lipase.

In summary, the data indicate that the AA of remnant acylglycerols is metabolized in Hep G2 cells by a lipoprotein receptor mediated uptake which is succeeded by liberation of AA from the acylglycerols, probably by lysosomal enzymes, and a preferential reincorporation into PC, PE and PI. Because considerable amounts of AA are secreted daily in rat bile and reabsorbed, this metabolic pathway may be important also for the transport of AA *in vivo*. Like the study of Habenicht *et al.* (40), who found that platelet-derived growth factor stimulated fibroblasts to metabolize AA of LDL to eicosanoids, this study stresses the potential physiological importance of the lipoprotein receptors in the transport of eicosanoid precursors.

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Myogenic Differentiation of the Muscle Clonal Cell Line BC3H-1 Is Accompanied by Changes in Its Lipid Composition

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Phospholipid and neutral lipid composition was studied in the course of myogenic differentiation of the clonal cell line BC3H-1. Total phospholipid content increased during differentiation, predominantly in the major classes of choline and ethanolamine glycerophospholipids. The contents of other lipids, such as triacylglycerols, diminished more than 50% during this period. The content and distribution of fatty acids also underwent marked differentiation-dependent changes. The polyunsaturated (tetrapenta- and hexaenoic) fatty acid species of several phospholipid classes diminished during differentiation, especially those in choline, serine and inositol glycerophospholipids. Most noticeable were the changes in phosphatidylserine; long-chain fatty acids having 20 to 22 carbon atoms and 4 to 6 double bonds decreased from about 30 to about 10 mol%. Although increased levels of saturation in other phospholipid fatty acyl chains appear to accompany the myogenic changes of BC3H-1 cells, some unsaturated fatty acids, such as oleic acid (18:1), increased by as much as 80% during the same period, suggesting the activation of a Δ9 desaturase. Sphingomyelin contained only saturated and monoenoic fatty acids and exhibited a four- to five-fold decrease in its content of monoenoic acyl groups. Diacylglycerols became enriched in arachidonate and docosahexaenoate. The amount of cholesterol and its esters increased slightly during differentiation of BC3H-1 cells. The data show that several metabolic pathways change during myogenic differentiation of the BC3H-1 clonal cell line, particularly *de novo* biosynthetic pathways, elongation/desaturation reactions, and acyl chain turnover. As a consequence of this, the lipid composition of the myoblast form of the BC3H-1 cell, in which the nicotinic acetylcholine receptor and other cell surface receptors are expressed, is thus different from that of the nondifferentiated cell.
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The nicotinic acetylcholine receptor (AChR) is one of the best characterized cell surface receptors (reviewed in ref. 1). The role of lipids in the maintenance of the structure and function of the AChR is becoming increasingly apparent (reviewed in refs. 2 and 3). The AChR is sensitive to the chemical nature of its lipid environment, and this has been experimentally demonstrated in *in vitro* studies in which the protein was reconstituted in lipid vesicles of different compositions, varying in the proportions of synthetic phospholipid species, fatty acyl chain lengths and degree of unsaturation, and in phospholipid/cholesterol ratio (4–6). With this strategy, the need for cholesterol and negatively charged phospholipids in i) the preservation of ligand-induced affinity transitions of the AChR and ii) the ability of the protein to translocate ions, *i.e.*, its gating property, was clearly established (4–5). The notion of the functional dependence of AChR on its lipid milieu has been complemented, and its relevance reinforced, by the discovery of an "annulus" of immobilized lipid molecules on the hydrophobic surface of the AChR protein as established by electron spin resonance techniques (reviewed in ref. 3).

As a natural extension of our *in vitro* studies, it is one of the aims of our current research to define *in situ*, in intact cellular systems, the chemical and physical characteristics of the lipids that constitute the immediate surrounding of the nicotinic AChR protein and those which form the rest of the bilayer, and also to study *in situ* the effect of lipids on the ligand binding and channel gating properties of the AChR. The clonal muscle cell line BC3H-1, originally derived by Schubert *et al.* (7) from a nitrosoethylurea-induced mouse cranial neoplasm, is a useful model toward this goal because lipid composition can be studied before and after cell surface expression of the AChR, one of the processes accompanying differentiation of the BC3H-1 cells (8). In addition, chemical changes which may occur can be correlated with the functional properties of the plasmalemma-assembled protein (Bouzat, C.B., Politi, L.E., Pediconi, M.F., de los Santos, E.B., and Barrantes, F.J., manuscript in preparation). In the present study, the content and composition of glycerophospholipids (neutral and polar classes), cholesterol and its esters (free and esterified forms of this sterol) and sphingomyelin were analyzed before and after cell differentiation. The results show a significant diminution in the content of neutral glycerolipids (triacylglycerols), an increase in various glycerophospholipids, predominantly the major classes of choline and ethanolamine glycerophospholipids, and changes in the content and distribution of their constituent fatty acids, which may be relevant to the structural and functional properties of the AChR and/or other membrane proteins whose expression changes in the course of cell differentiation.

MATERIALS AND METHODS

Materials. Fetal calf serum was purchased from CEVAN (Buenos Aires, Argentina).

Cell culture. Cells from the clonal cell line BC3H-1 (7) were grown unless otherwise specified in either 100-mm plastic Petri dishes or glass flasks at 37°C, essentially as described by Patrick *et al.* (8), in Dulbecco modified Eagle's medium containing 10% fetal calf serum in a Heraeus Cytoperm incubator maintained at 37°C with a 5% CO_2/95% air mixture. After the cultures reached confluency, the serum concentration was lowered to 0.5% to accelerate differentiation. Cells were analyzed 4–5 days thereafter. Differentiation was assessed by the changes in morphology (ref. 8, see also Fig. 1) and by the appearance of nicotinic AChR protein as assessed by binding assays using [^{125}I]α-bungarotoxin as described elsewhere (8). The maximum number of binding sites before and at

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Abbreviations: AChR, acetylcholine receptor; DMA, dimethylacetals; FFA, free fatty acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PS + CDP-DAG, phosphatidylserine plus cytidine diphosphate-diacylglycerol; PPI, polyphosphoinositides; Sph, sphingomyelin; TAG, triacylglycerol; TLC, thin-layer chromatography.

day-4 after differentiation were nil and 1.20 ± 0.10 pmol/mg protein, respectively.

Lipid extraction, isolation and quantitation. Cells were homogenized in a Potter-Elvehjem (Thomas type) homogenizer with chloroform/methanol (2:1, vol/vol) and extracted with this solvent mixture for 2–3 h at 20°C. The crude extracts were subsequently centrifuged, and the supernatants were washed according to the method of Folch *et al.* (9). The protein residues were then extracted with acidified solvents (10) to ensure complete recovery of polyphosphoinositides. The acidic extracts were washed and neutralized as described elsewhere (11). Phosphatidic acid (PA), phosphatidylserine + cytidinediphosphate-diacylglycerol (PS + CDP-DAG), phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were isolated by two-dimensional thin-layer chromatography (TLC) (12) of the neutral extracts. Plasmenyl-PC and plasmenyl-PE were determined as described previously (13). Polyphosphoinositides were separated from neutral and from acidic extracts by means of TLC on potassium oxalate-impregnated plates of silica gel H using the solvent system described by Shaik and Palmer (14). Standards of phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-*bis*phosphate (PIP_2) (Sigma, St. Louis, MO) were chromatographed in parallel with the samples. Neutral lipids such as triacylglycerols (TAG), diacylglycerols (DAG), and free fatty acids (FFA) were separated on silica gel G plates (Merck, Darmstadt, Germany) using hexane/diethyl ether/acetic acid (70:30:2, vol/vol/vol) as solvent system.

Transmethylation and gas-liquid chromatographic analysis of fatty acid derivatives. Thin-layer plates were sprayed with 2′,7′-dichlorofluorescein and viewed under ultraviolet light. The fractions were scraped into tubes containing eicosanoic acid as internal standard for gas-liquid chromatography. Fatty acid methyl esters were prepared according to the method of Morrison and Smith (15), using boron trifluoride (14% in methanol; Sigma) and subjected to gas-liquid chromatography using two (2 m × 0.2 cm inner diameter, 80–100 mesh) Chromosorb W-A/W columns (Supelco, Bellefonte, PA), in a Varian (Palo Alto, CA) 3700 chromatograph. The columns were connected to flame-ionization detectors operated in the dual-differential mode. The initial and final oven temperatures were 160°C and 220°C, respectively, and the rate of increase was 5°C/min. Injector and detector temperatures were 220°C and 230°C, respectively, and N_2 (30 mL/min) was the carrier gas. Chromatograms were quantitated with a CDS-111 Varian integrator. Peaks were identified by comparison of retention times with those of standards.

Other analytical methods. Total cholesterol content was determined by an enzymatic-colorimetric method (Wiener Laboratories, Rosario, Argentina). The method is based on the action of cholesteryl ester hydrolase, cholesterol oxidase, and a peroxidase coupled to a colorimetric assay. The content of free cholesterol was determined by a modification of the above-mentioned method using a mixture of enzymes without cholesteryl ester hydrolase. Phospholipid phosphorus was determined after treatment with perchloric acid of an aliquot of the total lipid extract or from the spots of the phospholipids isolated by two-dimensional TLC (12). Total protein content of the same samples was determined by the method of Lowry *et al.* (16) using bovine serum albumin as standard. Statistically significant differences were determined by the Student's *t*-test (two tailed).

Scanning electron microscopy. BC3H-1 cells were fixed overnight at 4°C in 0.37% formaldehyde and 0.5% glutaraldehyde in tissue culture medium. The cells were then washed with 0.1 M phosphate buffer, pH 7.2, and dehydrated with 75% acetone and 100% acetone (3 × 10 min each). The tissue was then incubated with Freon 113 diluted 1:3, 2:3 and 3:1 (vol/vol/vol) with acetone (15 min in each solution), in order to further dehydrate the tissue in a critical point chamber (Polaron, Watford, England) for 1 h. Samples were subsequently metalized with gold (200 Å) in a sputter coater model 3 (Pelco, Ted Pella Inc., Tustin, CA). The tissue was finally oriented for observation with a scanning electron microscope (JEOL 35 CF, JEOL, Tokyo, Japan) at 5 kV. Photographs were taken using Kodak SO-163 electron image film (Kodak, Rochester, NY).

RESULTS

Cholesterol and phospholipid distribution in BC3H-1 cells before and after differentiation. The muscle clonal cell line BC3H-1 undergoes marked morphological and biochemical changes during differentiation. One manifestation of the developmental changes is the expression of cell surface receptors, including the nicotinic AChR (8). This protein is absent from the plasmalemma at the predifferentiated stage, in which the cell exhibits a fibroblast-like morphology and reaches levels of about 300 fmol [^{125}I]α-bungarotoxin sites/35 mm dish at day 3 of differentiation, when the cell has already acquired the spindle-shaped, elongated, typical muscle-like morphology (Fig. 1). The maximum number of specific binding sites (1.20 ± 0.10 pmol/mg protein), as assessed by the [^{125}I]α-bungarotoxin assay, was reached at day-4 after differentiation, whereas no specific binding sites were detected 12 to 24 h after cell plating.

When the total phospholipid and cholesterol content in the BC3H-1 cells was measured at these two stages, we found that phospholipid content increased by about 40% upon differentiation (Table 1). Cholesterol and cholesteryl ester levels apparently increased by the same proportion as did phospholipids, but the increase was found not to be statistically significant (Table 1).

Further differences between non-differentiated and differentiated BC3H-1 cells became apparent when comparing the content and composition of individual phospholipids (Fig. 2). The major phospholipids of BC3H-1 cells, choline and ethanolamine glycerophospholipids, increased by 50% and 40%, respectively, after differentiation. Acidic phospholipids such as phosphatidylserine (PS), PI and sphingomyelin (Sph) did not change significantly during the same period.

Polyphosphoinositides (PPI) were found in quantities comparable to those of other cultured cells; PIP and PIP_2 represented less than 10% of the total phosphoinositide pool (Fig. 2). The BC3H-1 differentiated cells are thus relatively richer in phospholipids than the non-differentiated ones, with the two major classes (PC and PE) increasing most significantly.

Fatty acid composition of phospholipids in BC3H-1 cells. The fatty acid compositions of PC, PE and Sph are shown in Table 2 while those of PS and PI are presented in Table 3. PC exhibited characteristically very high

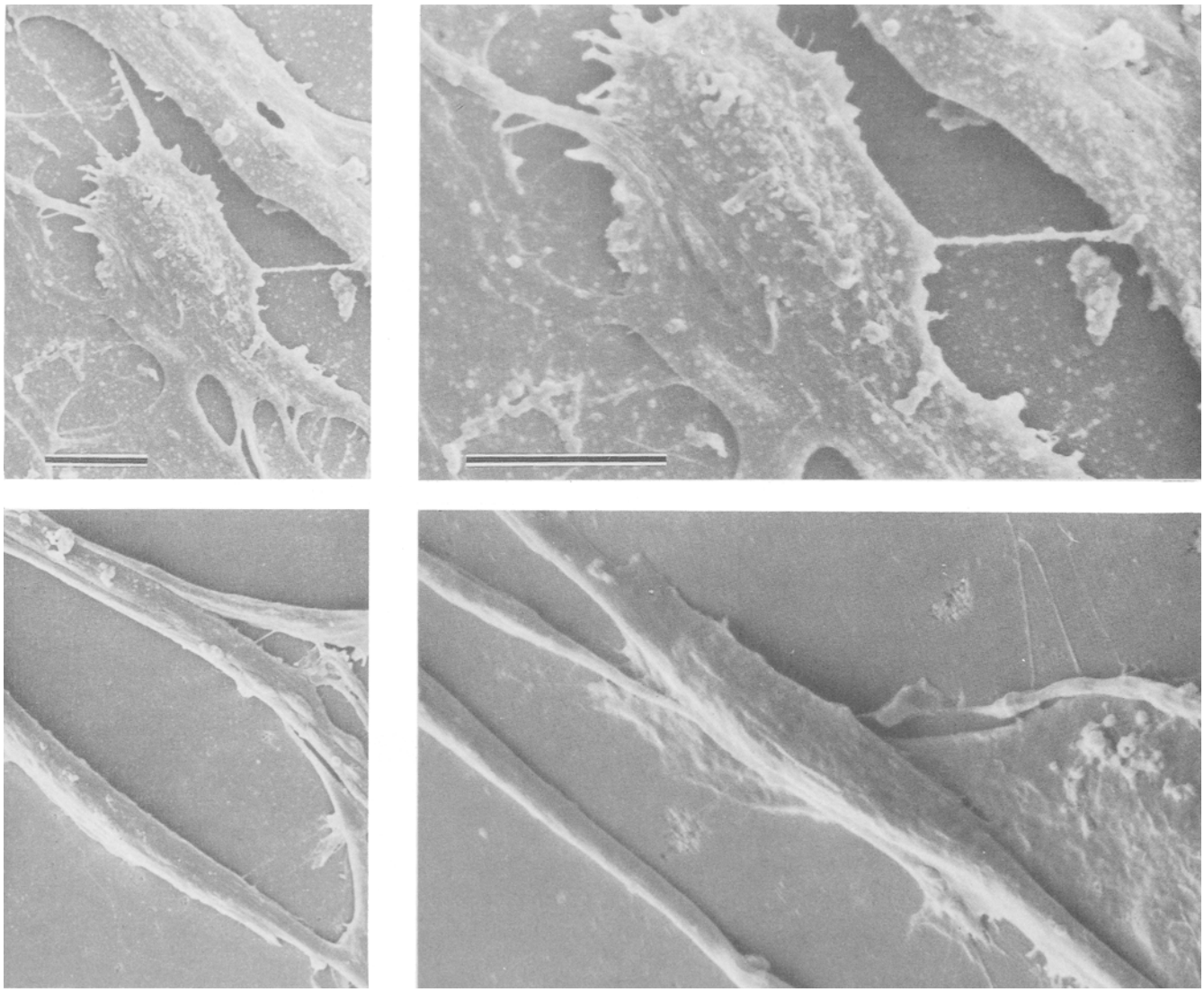

FIG. 1. Scanning electron micrographs of BC3H-1 cells before (upper panel) and four days after (lower panel) differentiation. The bars indicate 10 μm.

TABLE 1

Phospholipid and Sterol Content of BC3H-1 Cells[a]

Lipid	Undifferentiated	Differentiated
Phospholipids (n=5)	268.0 ± 25.0	369.0 ± 38.0[b]
Cholesterol (n=3)	94.8 ± 21.0	124.5 ± 14.1
Cholesteryl esters (n=3)	31.8 ± 11.2	42.9 ± 10.8
Cholesteryl ester/ cholesterol ratio	0.34	0.34
Phospholipid/cholesterol ratio	2.83	2.96

[a]Data are the mean ± SD, expressed in nmol lipid/mg protein. The molecular weight of cholesteryl oleate (651.1) was used as the average molecular weight for all cholesteryl esters.
[b]Statistically significant differences ($P < 0.005$).

saturated/unsaturated fatty acid ratios before differentiation, mainly due to the increased percentage of palmitate. Only 11% of the polyunsaturated long-chain fatty acids (C_{20}-C_{22}) were found before differentiation. Furthermore, the percentage of polyunsaturated fatty acids decreased by about 50% thereafter.

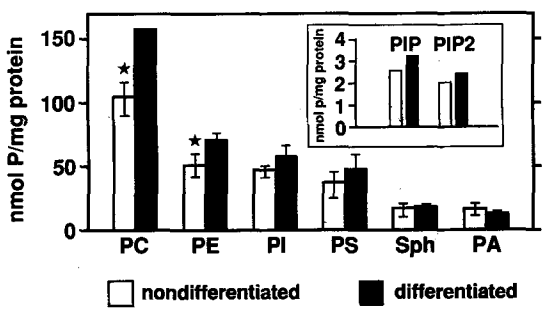

FIG. 2. Phospholipid content of BC3H-1 cells. Data represent the content of phospholipid P (nmol P/mg protein) of phospholipid classes expressed as mean value ± SD of six individual samples from two different experiments. Values for PIP and PIP_2 are from pooled samples (three samples each) from two different experiments. The lipids were extracted according to Folch *et al.* (9) and Uma and Ramakrishnan (10). Major phospholipids and PPI were isolated by the methods of Rouser *et al.* (12) and Shaik and Palmer (14), respectively. The size of the individual phospholipid pools was determined by measuring the lipid P content in each chromatographic spot.

TABLE 2

Fatty Acid Composition of Phosphatidylcholine, Phosphatidylethanolamine and Sphingomyelin During the Course of Differentiation of BC3H-1 Cells[a]

Fatty acid	Phosphatidylcholine			Phosphatidylethanolamine				Sphingomyelin			
	Undifferentiated	Differentiated		Undifferentiated	Differentiated			Undifferentiated	Differentiated		
		A	B		A	B			A	B	
14:0	1.9 ± 0.6	2.6 ± 0.7	1.2	3.0 ± 0.7	2.4 ± 0.2	2.6	14:0	6.4 ± 1.2	9.2 ± 1.6[b]	10.1	
16:0	29.9 ± 4.0	32.5 ± 0.8	28.8	16.1 ± 3.5	20.1 ± 1.5	20.4	16:0	24.5 ± 3.9	49.4 ± 4.4[b]	47.2	
16:1	9.0 ± 0.3	8.5 ± 2.3	11.2	5.8 ± 1.2	6.8 ± 0.9	7.6	16:1	10.8 ± 2.0	11.7 ± 1.8	12.4	
18:0	11.3 ± 0.8	10.5 ± 2.5[b]	16.2	21.5 ± 0.4	22.2 ± 1.3	20.5	18:0	9.9 ± 1.8	9.1 ± 2.1	9.7	
18:1	33.4 ± 0.7	36.0 ± 3.4[b]	30.7	23.8 ± 0.6	20.3 ± 1.7[b]	28.8	28.8	24.5 ± 3.4	8.2 ± 2.3[b]	7.6	
18:2n-6	2.5 ± 0.2	1.3 ± 0.2	2.7	1.2 ± 0.4	1.7 ± 0.4	1.6	20:0	3.0 ± 0.7	3.1 ± 1.0	4.5	
18:3/20:1	0.7 ± 0.3	1.3 ± 0.3	1.1	0.8 ± 0.3	0.8 ± 0.3	1.2	21:0	1.5 ± 0.6	1.0 ± 0.2[b]	0.7	
20:3	0.8 ± 0.2	0.9 ± 0.2	0.9	0.9 ± 0.1	0.6 ± 0.2[b]	0.7	22:0	1.4 ± 0.4	2.1 ± 0.3	1.9	
20:4n-6	4.3 ± 0.8	3.4 ± 0.5	2.9	8.5 ± 1.2	11.6 ± 1.8[b]	8.0	23:0	1.0 ± 0.3	2.4 ± 0.4	2.0	
20:5n-3	0.8 ± 0.3	0.9 ± 0.3	1.2	2.1 ± 0.5	1.5 ± 0.6	1.1	24:0	1.1 ± 0.4	0.5 ± 0.4	0.8	
22:4n-6	1.1 ± 0.2	0.8 ± 0.1[b]	1.3	4.0 ± 0.8	1.6 ± 0.3[b]	0.5	24:1	5.5 ± 1.3	1.1 ± 0.8[b]	1.0	
22:5n-6	1.9 ± 0.5	0.2 ± 0.0[b]	0.4	5.6 ± 0.4	4.7 ± 0.2[b]	3.0	25:0	1.9 ± 0.3	0.8 ± 0.4[b]	1.1	
22:5n-3	2.3 ± 0.7	0.7 ± 0.3[b]	0.8	6.7 ± 0.6	5.8 ± 0.7	3.1	26:0	8.5 ± 2.0	1.0 ± 0.4[b]	0.9	
22:6n-3	0.4 ± 0.1	0.4 ± 0.1	0.6	0.2 ± 0.1	0.2 ± 0.1	0.8					
Saturated/ unsaturated ratio	0.76	0.86		0.68	0.80	0.77	Saturated/ unsaturated ratio	1.5	3.67	3.74	

[a]Data are mean ± SD (mol%) of five samples from two different experiments for undifferentiated cells and cells differentiated in 0.5% fetal calf serum (condition "A"), and of two samples for cells grown in 10% fetal calf serum (condition "B") in the medium. Values corresponding to sphingomyelin are from three separate samples.

[b]Statistically significant differences ($P < 0.05$).

Phosphatidylethanolamines displayed a unique feature among the phospholipid classes in that they maintained the same hydrocarbon chain composition during differentiation. As a consequence, PE showed approximately the same proportion of long-chain fatty acids with four or six double bonds (the sum of these acyl groups is 27.2 and 25.4 mol% in non-differentiated and differentiated cells, respectively). Very low levels of docosapentaenoate and docosahexaenoate were found in PE with respect to the total fatty acyls (Table 2).

Among the minority lipids, Sph displayed a fatty acid profile exclusively made up of saturated and monoenoic species, which accounted for its low degree of unsaturation (Table 2). Sph was one of the lipid classes exhibiting considerable changes in the proportion of saturated and monoenoics after differentiation (0.7 at the initial, undifferentiated stage and 2.1 after differentiation). Sph was mainly esterified with palmitic and oleic acids; 24:1 (which predominates in this lipid in mammalian tissues) was only the third or fourth most important fatty acid in BC3H-1 cells. The marked decrease in the content of 26:0 after differentiation (from 8.5 to 1.0 mol%, Table 2) is also noteworthy.

The analysis of acyl groups present in PS and PI also showed drastic changes in polyunsaturated fatty acids in the course of myogenic differentiation (Table 3). The results reveal that in addition to the increase in saturated fatty acids, such as palmitate (more than a two-fold increase), long-chain fatty acids having 20 to 22 carbon atoms and 4 to 6 double bonds decreased from about 30 to about 10 mol%. This constituted the most important change in acyl group content detected for all phospholipids analyzed. Thus, although increased levels of saturation in phospholipid fatty acyl chains appear to accompany the myogenic differentiation of BC3H-1 cells, some unsaturated species, such as oleic acid, increase by as much as 80% during the same period, suggesting a compensatory mechanism involving the activation of a Δ9 desaturase. Before BC3H-1 cell differentiation, PI has a characteristic fatty acid profile as found in mammalian tissue, with stearate and arachidonate accounting for more than 50% of the total. PI exhibited variations different to those observed with other glycerophospholipids: no increases in saturated fatty acids (16:0 and 18:0) were apparent; after cell differentiation, however, the relative proportion of oleic (18:1) and arachidonic (20:4) fatty acyl moieties changed noticeably (Table 3).

Differentiation of BC3H-1 cells is a reversible process which can be induced either by removal of growth factors from the culture medium or by allowing cells to reach confluency. The most commonly used procedure consists of decreasing the serum concentration in the culture medium, thus eliminating the inhibition exerted by the serum on protein synthesis and cell proliferation (17). In order to assess whether the changes in fatty acid composition of the cells were determined by the lipids present in the culture medium, fatty acid analyses were carried out on the phospholipids extracted from cells differentiated with low (0.5%) and high (10%) fetal calf serum. In low (0.5%) calf serum, BC3H-1 cells attained differentiation within 3 to 4 days, as judged by i) the typical morphological changes (cf. Fig. 1) and ii) the appearance of α-bungarotoxin sites on the plasmalemma of intact cells (see above). The results presented in Tables 2 and 3 indicate that the

TABLE 3

Composition of Acyl Chains in Phosphatidylserine and Phosphatidylinositol During Differentiation of BC3H-1 Cells[a]

Fatty acid	Phosphatidylserine			Phosphatidylinositol		
	Undifferentiated	Differentiated		Undifferentiated	Differentiated	
		A	B		A	B
16:0	5.7 ± 0.5	12.7 ± 2.0[b]	10.4	9.2 ± 2.6	8.5 ± 1.8	8.7
16:1	1.9 ± 0.7	5.8 ± 2.1[b]	6.2	3.1 ± 1.6	3.1 ± 1.1	4.2
18:0	28.4 ± 2.6	27.4 ± 4.3	29.3	28.9 ± 5.6	24.5 ± 2.9	26.3
18:1	19.5 ± 1.8	35.3 ± 2.5[b]	33.6	17.5 ± 4.5	31.0 ± 5.2[b]	30.5
18:2n-6	6.3 ± 0.4	5.2 ± 1.0	4.5	5.2 ± 1.0	3.7 ± 0.2	4.3
18:3/20:1	5.5 ± 0.1	1.9 ± 0.3[b]	5.2	3.2 ± 0.3	3.6 ± 0.1	3.0
20:4n-6	10.3 ± 1.4	5.5 ± 1.3[b]	5.0	23.6 ± 4.5	12.1 ± 2.2[b]	14.0
20:5n-3	3.4 ± 0.2	1.0 ± 0.3	0.9	2.6 ± 0.6	2.2 ± 0.4	2.0
22:4n-6	3.5 ± 0.7	n.d.	n.d.	1.1 ± 0.3	2.4 ± 0.2[b]	1.8
22:5n-6	3.0 ± 0.6	1.7 ± 0.4	1.9	1.8 ± 0.3	1.2 ± 0.2	1.1
22:5n-3	4.8 ± 0.7	2.1 ± 0.5[b]	2.3	1.4 ± 0.4	2.8 ± 0.5	2.1
22:6n-3	7.7 ± 2.1	1.4 ± 0.4[b]	0.6	0.9 ± 0.3	2.3 ± 0.3[b]	2.0
Saturated/ unsaturated ratio	0.52	0.67	0.66	0.62	0.50	0.54

[a]Data are mean value ± SD (mol%) of five samples from two different experiments except those from B condition, which are mean values from two different samples. n.d., Not detected.
[b]Statistically significant differences ($P < 0.05$).

observed changes in the fatty acid composition of phospholipids in BC3H-1 cells are not due to the growth medium conditions but are related to the myogenic developmental change itself.

The contribution of plasmalogens to the total PE pool were 68 and 74%, while for PC plasmalogens constituted 55% and 51% of the total before and after differentiation, respectively. The composition of ether-linked aliphatic chains in PC and PE varied during the course of myogenic differentiation (Fig. 3). Mature cells became richer in 16:1 and 18:1 dimethylacetals in both PC and PE, while the proportion of 16:0 diminished significantly in both phospholipids.

Fatty acid composition of triacylglycerols, diacylglycerols and free fatty acids in BC3H-1 cells. Though small, the pools of acylglycerols play an important role as acyl group donors for fatty acid turnover. We thus investigated their content and composition in differentiating BC3H-1 cells. As shown in Table 4, triacylglycerols decreased by 50% during differentiation (11.2 ± 0.3 μg/mg protein before and 4.1 ± 0.1 μg/mg protein after, respectively). Diacylglycerols increased during this process (1.8 to 2.96 μg/mg protein), whereas free fatty acids remained constant (26 and 21 μg/mg protein before and after differentiation, respectively). Oleate, palmitate, stearate and linoleate accounted for more than 75% of all acyl chains in triacylglycerols. Palmitate, stearate and oleate were the main components in both diacylglycerols and free fatty acids. The 20:3n-9 decreased in triacylglycerols while it increased in diacylglycerol and free fatty acid pools during differentiation (Table 4).

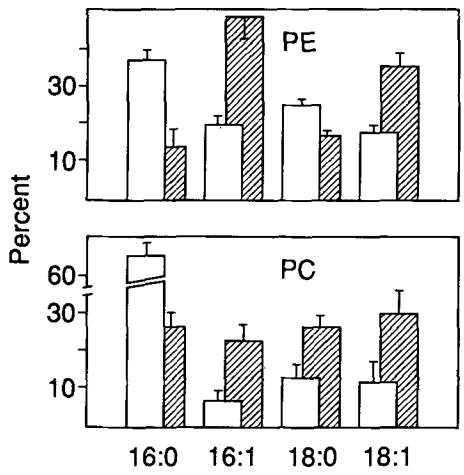

FIG. 3. Percentage distribution of ether-linked moieties in choline and ethanolamine glycerophospholipids. The relative contribution of C_{16} and C_{18} dimethylacetals found in PC and PE in undifferentiated (empty bars) and differentiated (dashed bars) BC3H-1 cells is shown. One-hundred percent corresponds to the sum of the content of the four DMA shown in the figure. Each bar represents the mean ± SD of determinations carried out in three different cultures. All differences are statistically significant ($P < 0.025$).

DISCUSSION

Important changes in the metabolism and expression of many proteins and other molecular constituents of cell membranes occur during cell differentiation. In BC3H-1 cells, differentiation-related changes involve the expression of a variety of muscle specific gene products, including the muscle form of the enzyme creatine kinase (7) and the AChR protein (8), among others. Cell membranes also exhibit substantial changes in absolute mass and turnover during cell differentiation (18,19). The developmentally-regulated appearance of insulin receptors and insulin-mediated effects on lipid metabolism in BC3H-1 cells (20) is a particularly clear manifestation of the importance of membrane phenomena associated with lipid metabolism in this nonfusing cell line. The overall lipid composition of the BC3H-1 cells reported here resembles that of other cell lines (21,22), and the changes undergone during differentiation in the majority of phospholipid

TABLE 4

Fatty Acid Composition of Triacylglycerols, Diacylglycerols and Free Fatty Acids from BC3H-1 Cells

	Triacylglycerols[a]		Diacylglycerols[b]		Free fatty acids[b]	
Fatty acid	Undifferentiated	Differentiated	Undifferentiated	Differentiated	Undifferentiated	Differentiated
14:0	1.9 ± 0.4	3.4 ± 0.8	2.2	2.4	3.6	1.6
16:0	19.1 ± 1.9	28.9 ± 0.1[c]	17.9	15.9	24.1	22.1
16:1	4.6 ± 0.4	6.8 ± 1.1[c]	6.1	10.7	8.5	3.7
18:0	16.4 ± 1.9	10.3 ± 0.4[c]	23.6	20.1	21.8	18.3
18:1	30.4 ± 3.7	32.5 ± 0.1	32.7	28.6	23.6	23.2
18:2n-6	11.4 ± 1.7	7.9 ± 2.2	2.9	2.6	5.6	7.1
18:3/20:1	n.d.	n.d.	n.d.	n.d.	4.5	6.0
20:3	8.5 ± 2.2	3.5 ± 0.2[c]	10.5	13.3	2.6	16.0
20:4n-6	2.2 ± 0.7	2.0 ± 0.4	0.1	0.9	0.4	0.3
20:5n-3	n.d.	n.d.	2.6	1.5	n.d.	n.d.
22:4n-6	n.d.	n.d.	n.d.	n.d.	0.3	0.1
22:5n-6	n.d.	n.d.	n.d.	n.d.	1.6	0.7
22:5n-3	2.0 ± 0.4	1.5 ± 0.4	0.1	0.1	1.7	0.6
22:6n-3	2.2 ± 0.3	3.2 ± 0.6	1.2	3.9	1.0	0.2
Saturated/ unsaturated ratio	0.60	0.74	0.78	0.62	0.98	0.72

[a]Values expressed as mol% are mean ± SD of three separate experiments; n.d., not detected.
[b]Three samples were pooled and the fatty acid composition was determined for free fatty acids and diacylglycerols from two experiments.
[c]Statistically significant differences ($P < 0.05$).

classes exhibit the same type of increases observed with other cells in culture (21–23). No relative changes in the phospholipid/cholesterol content were apparent (Table 1), in contrast with the variations in this ratio reported for some cells (23). The proportion of cholesteryl esters in BC3H-1 cells is, however, higher than that found in other AChR-containing cells such as electrocytes (24). The present work shows that during differentiation a net decrease in TAG and an increase in PL occurred. Similar observations were reported by Sauro and Strickland (25) in muscle cells. L6 myoblasts, but not differentiated L6 myotubes, accumulate large stores of neutral lipids, in accordance with previous findings showing the activation of a lysosomal triacylglycerol lipase after differentiation (26).

The most important differentiation-related changes in BC3H-1 lipids were found in the phospholipid fatty acid moieties. Phospholipids become richer in 18:1 and poorer in C_{20} and C_{22} polyenoic acids. Decreased levels of arachidonate, 22:4n-6, and 22:5 from both the n-3 and n-6 series, and 22:6n-3 have been reported for other transformed cell lines (19). Interestingly, neuroblastoma and astrocytoma established cell lines have been reported to display a decreased ability to elongate and desaturate long-chain fatty acids in comparison to normal brain cells (27). Tables 2 and 3 show that a net decrease on the polyunsaturated fatty acids takes place in BC3H-1 cells upon differentiation, especially in PS, PC and PI. In these cells, the desaturases may be subjected to different pressures in the undifferentiated and differentiated stages. Both 18:3 and 20:1 methyl esters co-migrate under the present chromatographic conditions. It is not possible, therefore, to unambiguously ascertain which is the fatty acid that increased in PC after differentiation (see Table 2). If this change were due to 18:3, as it is the precursor of other C_{20} and C_{22} polyunsaturated fatty acids, it would reflect the accumulation of this series precursor. The three- and five-fold decrease observed for 18:1 and 24:1, respectively, in Sph monounsaturated fatty acids suggests, however, that other metabolic pathways, specifically fatty acid turnover, may also be modified in the course of differentiation. In fact, the response of desaturases to dietary alterations appears to indicate that Δ6 and Δ5 desaturases are less dramatically influenced by fluctuations in the availability of dietary substrates than the Δ9 desaturase (28). The two former desaturases may be subjected to inhibitory mechanisms in differentiated, but not in undifferentiated, stages of BC3H-1 cell development.

The fatty acyl profile in neutral glycerides and free fatty acids remained relatively constant during differentiation. This is surprising if one considers these pools as a "reservoir" of fatty acid constituents for membrane phospholipids. Acyltransferases and various lipases, especially phospholipases, are responsible for the uptake, distribution and release of fatty acids from glycerophospholipids. The diminished amounts of long-chain polyunsaturated fatty acids in BC3H-1 phospholipids may be consistent with their reported deleterious effects on some types of cells in culture. For example, polyunsaturated fatty acids of the n-3 and n-6 series have been shown to inhibit the growth of a mammary adenocarcinoma and a leukemic T cell line, respectively (29,30). The relative composition of the fatty acid pool varied with differentiation of the BC3H-1 cells. Thus, eicosatrienoate increased about five-fold, pointing to the activation of a lipase or inactivation of an acyltransferase during development.

Physical consequences are likely to derive from the changes in the chemical composition of membranes occurring in the BC3H-1 cells. High proportions of 18:1 together with a low percentage of 20:4n-6 and C_{22} polyunsaturated fatty acids with six double bonds should be associated with a more "ordered" bilayer upon differentiation. Physical rearrangement of membrane lipids has also been reported to occur during differentiation of red blood cells (18). The immature cells contain regions of more disordered, loosely packed lipids, that are discarded after enucleation (31). A similar change may take place in

the BC3H-1 cell, with concomitant changes in membrane fluidity.

Previous studies from our laboratory (4,5; see also ref. 3 for review) demonstrated the essential need for some phospholipids such as PE, in combination with cholesterol, for the preservation of AChR functional properties *in vitro*. In the present work we have observed that the size and composition of phospholipid pools like that of PE increase or are maintained during differentiation of BC3H-1 cells. Also, the degree of saturation of this phospholipid, that plays an important role in the affinity states and ion translocation phenomena of the AChR (5,32), seems to be preserved in the differentiated BC3H-1 cell that expresses cell surface receptor. Cholesterol levels are also critical for the preservation of other AChR properties (4), and we report here that its level is maintained during differentiation (cf. Table 1). The cholesterol/cholesteryl ester ratio also remained constant upon differentiation of BC3H-1 cells (cf. Table 1). Electrophysiological work carried out since submission of the present paper (Bouzat, C.B., Politi, L.E., de los Santos, B.E., Pediconi, M.F., and Barrantes, F.J., unpublished results, and ref. 33) appears to confirm our working hypothesis; changes in the proportions and in the quality of the lipids in the AChR environment *in situ*, using the BC3H-1 model system, exert marked effects on the channel gating properties of this rapid ligand-operated receptor.

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Occurrence of Long and Very Long Polyenoic Fatty Acids of the n-9 Series in Rat Spermatozoa

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Dietary deficiency of essential fatty acids of the n-3 and n-6 series is known to promote a compensatory increase in polyenoic fatty acids of the n-9 series in the lipids of mammalian tissues. In the present study long-chain n-9 polyenes were found to be normal components of the epididymis and especially of sperm isolated from that tissue, in healthy, well-fed, fertile rats maintained on essential fatty acid-sufficient diets. The n-9 polyenes occurred in large concentrations in the choline glycerophospholipids (CGP), the major phospholipid class of spermatozoa in epididymal *cauda*, and were highly concentrated in plasmenylcholine, the major subclass of CGP. The uncommon polyene 22:4n-9 was found in the highest proportion, followed in order of relative abundance by 22:3n-9, 20:3n-9 and 24:4n-9. These polyenes were probably derived from oleate (18:1n-9) in much the same way as long-chain polyenes of the n-6 and n-3 series are derived from linoleate (18:2n-6) and linolenate (18:3n-3), respectively.

***Lipids* 27, 676-680 (1992).**

In the absence of dietary essential fatty acids of the n-6 and n-3 series, animals synthesize oleate (18:1n-9) derived polyenoic acids (1,2). The most familiar of these compounds is 20:3n-9, first identified by Mead and Slaton (3), and known to be formed from 18:1n-9 when there is not sufficient 18:2n-6 in the diet to synthesize ample 20:4n-6. Rats given diets having hydrogenated coconut oil as the only source of fat for five months accumulated 20:3n-9 in place of 20:4n-6 in erythrocytes and produced small, but measurable amounts of two minor fatty acids behaving chromatographically as 22:3n-9 and 22:4n-9 (4). Holman (1) demonstrated that competitive interactions occur such that n-3 acids suppress the metabolism of n-6 polyenes, n-6 acids suppress the metabolism of n-3 polyenes less strongly, and both suppress the formation of long-chain n-9 polyenes. Brenner and Peluffo (5) showed that the desaturase acting on 18:1n-9 in liver is inhibited by 18:2n-6 and 18:3n-3. These studies support the widely accepted view that long-chain n-9 polyenes do not normally accumulate in mammalian tissues except under n-6 and n-3 polyenoic fatty acid deficient conditions.

In the present paper an unexpected departure from this view is described. Large amounts of long-chain n-9 polyenes were found as normal acyl groups of a major lipid in essential fatty acid sufficient, healthy laboratory rats. The fatty acids in question occur in the epididymal tissue and are highly concentrated in the spermatozoa isolated from the caudal region of the epididymis.

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Abbreviations: CGP, choline glycerophospholipid; DMDC, dimethyldicarboxylate; EGP, ethanolamine glycerophospholipids; FAME, fatty acid methyl esters; GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Male Albino Wistar rats (weight, 350-370 g) were killed by decapitation, the epididymal tissue was carefully dissected, and the proximal (*caput*) and distal (*cauda*) sections were isolated. Spermatozoa were prepared essentially as previously reported (6,7), by cutting the epididymal sections into small pieces, gently stirring the pieces in a salt-containing suspension medium (pH 7.4), and filtering through a fine mesh cloth. After several washings, the sperm cells were collected from the filtrates by centrifugation. Lipid extracts from whole epididymis or from spermatozoa were prepared and washed using the procedures described by Folch *et al.* (8). Phospholipids were resolved into classes by thin-layer chromatography (TLC) (9) and the diradyl-choline and -ethanolamine glycerophospholipids (CGP, EGP) from the epididymal *cauda* were eluted from the absorbent with chloroform/methanol/acetic acid/water (50:39:1:10, by vol) (10) for further analyses. A portion of each lipid fraction was exposed for 1 min to 0.5N HCl in chloroform/methanol (2:1, vol/vol), which released the aldehydes from the *sn*-1 position of the plasmenyl subclasses, yielding lysophosphatidylcholine and lysophosphatidylethanolamine. The lyso derivatives were separated from the unreacted lipids, mostly phosphatidylcholine and phosphatidylethanolamine, by TLC for fatty acid analysis.

Fatty acid methyl esters (FAME) were prepared by transesterification with BF_3/methanol (11). FAME were separated into fractions according to unsaturation by argentation TLC (using silica gel G/$AgNO_3$, 5:1, w/w, with chloroform/methanol, 95:5, vol/vol as solvent). FAME were then analyzed by gas-liquid chromatography (GLC) on glass columns (2 m × 2 mm i.d.) packed with 15% OV-275 on WAW, 100-200 mesh (Varian, Sunnyvale, CA), with nitrogen as the carrier gas. For fatty acid compositional analyses, a linear (5°C/min) temperature program was employed, the initial and final column oven temperatures were 160 and 220°C, respectively. Injector and flame ionization detector temperatures were 220 and 230°C, respectively, and the electrometer was operated in the dual-differential mode.

Unsaturated FAME fractions in methanol were hydrogenated by bubbling H_2 in the presence of palladium oxide through the solution (12). After adding water, the saturated FAME were recovered in hexane and analyzed by GLC. Oxidative ozonolysis was done by bubbling O_3 into ice-cold BF_3/methanol solutions of FAME (13). Water was added and the products were extracted with a small volume of hexane and subjected to TLC (hexane/diethyl ether, 95:5, vol/vol). This separated the methyl ester derivatives of monocarboxylic from those of dicarboxylic acids (the main products of fragmentation of the fatty acid molecules at their double bonds under these conditions). The products were recovered from the silica gel by adding a small volume of water and partitioning

with a small volume of CS_2, the solvent in which all samples were injected into the gas chromatograph.

The food pellets (Purina Lab Chow) routinely fed to the rats were analyzed by GLC. Nearly 40% of the total fatty acids in the diet was 18:2n-6, 24.4% was 18:1n-9 and 4.6% was 18:3n-3. Other fatty acids were 18.5% 16:0, 2.2% 16:1 and 5.4% 18:0. Variations in the 18:2/18:1 ratio in the diet did not significantly affect the fatty acid composition of the major sperm lipids.

RESULTS

Analysis of the fatty acids of rat epididymal and spermatozoal lipids showed abundant proportions of unfamiliar long-chain polyunsaturated components. These compounds, in parallel with known n-6 and n-3 polyenes, were subjected to a series of analytical tests to establish their identity (Fig. 1). When resolved by argentation TLC, the unusual fatty acids migrated as trienoic and tetraenoic FAME. Under both isothermal and temperature-programmed GLC conditions (Fig. 1, panels A and B), their elution behavior corresponded to that of 20, 22 and 24 carbon polyenes. The fatty acids tentatively identified as 20:3n-9, 22:3n-9, 22:4n-9 and 24:4n-9 always eluted ahead of the corresponding n-6 isomers, as would be expected from a polar stationary phase, as the one used in this experiment. When catalytic hydrogenation was performed on portions of the tri-, tetra- and pentaenoic FAME fractions, only 20-, 22- and 24-carbon saturated products were obtained, thus confirming the assigned chain lengths.

The trienoic and tetraenoic FAME fractions isolated from *cauda* sperm plasmenylcholine by argentation TLC were used as the sources of 22:3 and 22:4, respectively, to establish the position of the double bonds in both fatty acids. Ozonolysis of an unsaturated fatty acid in the presence of ice-cold BF_3/methanol (13) oxidatively cleaves the double bonds to yield (the methyl esters of) a monocarboxylic and a dicarboxylic acid whose number of carbons is diagnostic of the position of the double bond(s) in the original compound. For example, dimethyl azelaidate (C_9) was, as expected, the main dimethyldicarboxylate (DMDC) formed from 18:1n-9 and from 18:2n-6 (see Scheme 1). Arachidonate (20:4n-6), in turn, yielded low but

$$CH_3\text{-}(CH_2)_7\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}COOCH_3 \rightarrow$$

$$CH_3\text{-}(CH_2)_7\text{-}COOCH_3 + H_3COOC\text{-}(CH_2)_7\text{-}COOCH_3$$

SCHEME 1

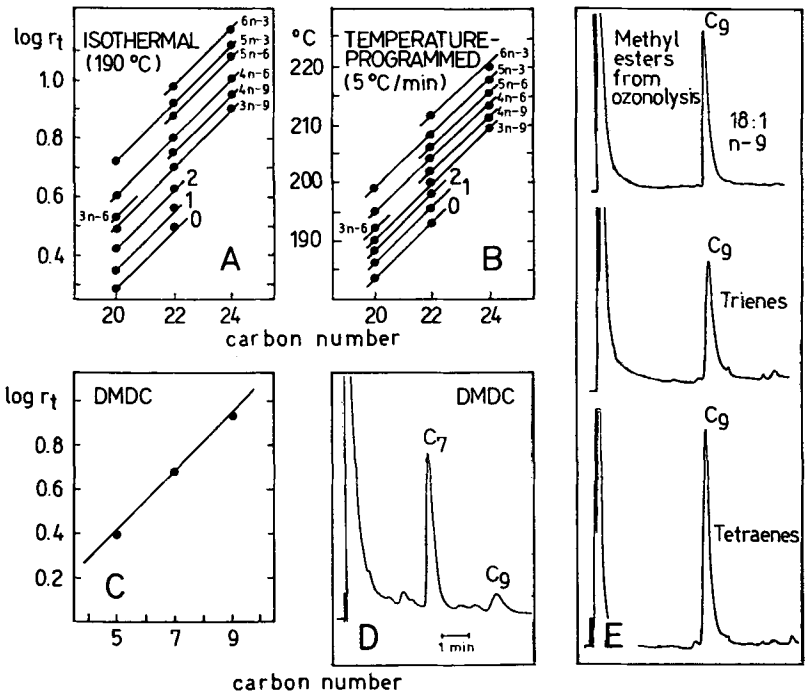

FIG. 1. Gas-liquid chromatographic analysis of FAME and of the fragments produced after oxidative ozonolysis of the trienoic and tetraenoic fatty acids from rat sperm plasmenylcholine. Glass columns packed with a polar stationary phase (OV-275, Varian) were used. A, Logarithms of retention times (r_t) of FAME as a function of their number of carbons under an isothermal condition. B, Elution temperatures, using a linear temperature program, *vs.* the number of carbons in the same compounds. C, Behavior of DMDC obtained after ozonolysis of known fatty acids (20:4n-6, 22:5n-3 and 18:1n-9) under isothermal conditions (120°C). D, DMDC obtained from the trienoic fraction of rat sperm plasmenylcholine. E, GLC of the short-chain methyl ester derivatives (temperature programmed at 10°C/min, with an initial temperature of 50°C) obtained after ozonolysis of oleate and of the trienoic and tetraenoic fractions from rat spermatozoa plasmenylcholine. The product was almost exclusively methyl nonanoate in all cases.

detectable amounts of the expected C_5 derivative, and 22:5n-3 [previously isolated from mammalian (bovine) retina to be used as a standard] gave the predictable C_7 DMDC (Fig. 1, panel C). These served for comparison of the products formed after ozonolysis of the FAME from rat sperm plasmenylcholine. The major DMDC from its trienoic FAME fraction had seven carbons (Fig. 1, panel D), thus allowing identification of the fatty acid parent as 7,10,13-docosatrienoate, or 22:3n-9.

Naturally occurring fatty acids having four carbons between the carboxyl end and their first double bond, such as 22:6n-3, 22:5n-6 and 22:4n-9 (4,7,10,13,16,19-docosahexaenoate, 4,7,10,13,16-docosapentaenoate and 4,7,10,13-docosatetraenoate, respectively) yield a four carbon DMDC from the carboxyl end and a C_3, C_6 and C_9 methyl ester, respectively, from the methyl end (in addition to dimethyl malonate). Small amounts of the C_4 DMDC were produced from the 22:4n-9 from rat sperm, probably because the present conditions were not satisfactory for the analysis of short-chain volatile derivatives. However, considering the other main product of ozonolysis, virtually the only methyl ester produced from the plasmenylcholine tetraenoic fraction was methyl nonanoate (Fig. 1, panel E). This was also the main methyl ester produced by ozonolysis from the trienoic fraction, confirming the presumption that the major polyenoic fatty acids of both fractions belonged to the oleic acid series. Thus, the major trienoic and tetraenoic components of plasmenylcholine from *cauda* spermatozoa could be conclusively identified as 22:3n-9 and 22:4n-9 (7,10,13-docosatrienoic and 4,7,10,13-docosatetraenoic acids, respectively).

The fatty acid composition of each of the two main phospholipid classes, EGP and CGP (together totaling about 80% of the total phospholipids) of *caput* and *cauda* epididymis differed significantly from that of spermatozoa isolated from such regions (Fig. 2). In both stages of maturation, sperm cells were much richer in fatty acids with longer chains (C_{22}, C_{24}) and with more double bonds (four, five) than the corresponding epididymal regions. The percentages of 22:3n-9 and 22:4n-9 were several-fold larger in CGP of caudal epididymal spermatozoa than in CGP from *cauda* epididymis (Table 1), showing that sperm cells were the main contributors to the n-9 polyenes observed in the latter epididymal region. Interestingly, the opposite was true for the EGP, in which the percentages of the same acyl chains were lower in spermatozoa than in the corresponding epididymal regions.

The fatty acid compositions of the two major lipids of spermatozoa also were affected differently by epididymal maturation. Comparing the sperm cells from *caput vs.* those from *cauda* regions, it was clear that their epididymal progression resulted in a significant increase in the percentage of long (C_{22} and C_{24}) polyenoic fatty acids of the n-9 series (Fig. 2). Such an increase was specifically observed in CGP (from ~3% to ~25% of the total fatty acids of this phospholipid). Since CGP represented nearly 50% of the total phospholipid of spermatozoa, this change in fatty acids significantly affected the fatty acid composition of the spermatozoon. In fact, when the total fatty acids of sperm were analyzed, the sum of the n-9 polyenes was observed to change from less than 2% in *caput* to about 12% in *cauda* sperm (data not shown), demonstrating that CGP was the major contributor of such fatty acids. Consistently n-9 polyenes were either minor components or virtually absent from other lipid classes. An interesting exception was phosphatidylinositol, which accounted for only 4% of the total phospholipid, but contained more than 15% n-9 polyenes (14).

The plasmalogens of EGP and CGP of both epididymis and spermatozoa were richer than the corresponding phosphatidyl subclasses in n-9 polyenes (Table 1). The contribution of plasmalogens to the total EGP and CGP in spermatozoa differed from that in the corresponding epididymal regions. Thus, plasmenylethanolamine and plasmenylcholine in *cauda* epididmis accounted for

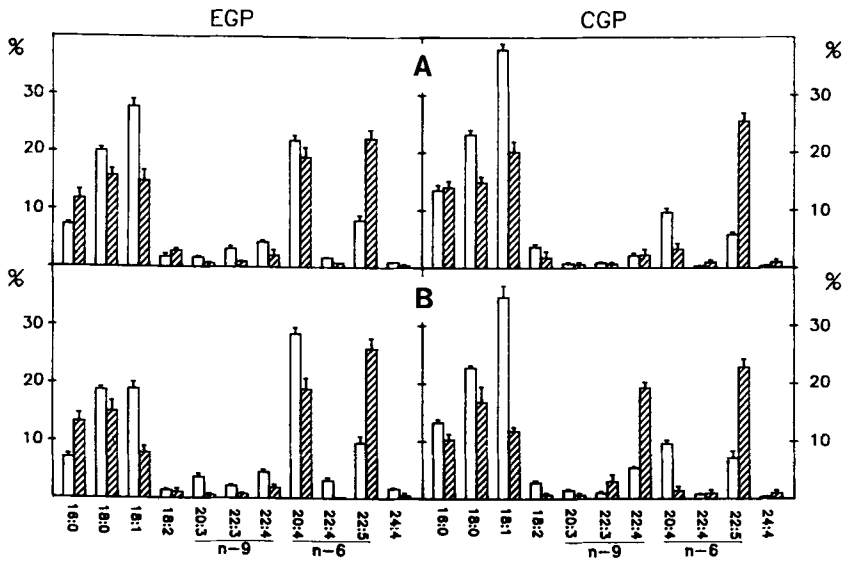

FIG. 2. Fatty acid composition of EGP and CGP from epididymal tissue (open bar) and epididymal spermatozoa (hatched bar). A, *caput*; and B, *cauda*. Phospholipids were isolated by TLC and fatty acids (as methyl esters) were analyzed by GLC. Results are given as percentages of the total fatty acids in each lipid (mean values ± SD from three and four individual samples for epididymal tissue and spermatozoa, respectively).

TABLE 1

Fatty Acids of Major Subclasses of Ethanolamine and Choline Glycerophospholipids from *Cauda* Epididymis and Epididymal Spermatozoa[a]

	Phosphatidylethanolamine		Plasmenylethanolamine		Phosphatidylcholine		Plasmenylcholine	
	Epididymis	Spermatozoa	Epididymis	Spermatozoa	Epididymis	Spermatozoa	Epididymis	Spermatozoa
16:0	10.1 ± 1.0	23.7 ± 1.5	1.6 ± 0.3	1.9 ± 0.3	14.2 ± 0.5	15.8 ± 0.8	4.7 ± 0.2	1.4 ± 0.2
16:1	0.7 ± 0.1	—[b]	0.5 ± 0.3	1.0 ± 0.1	0.2 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.4 ± 0.1
18:0	27.8 ± 0.8	16.6 ± 0.3	2.8 ± 0.6	2.2 ± 0.1	24.3 ± 0.4	26.4 ± 0.4	7.3 ± 0.5	1.5 ± 0.2
18:1	23.2 ± 1.4	6.4 ± 0.3	11.6 ± 0.8	8.1 ± 0.4	36.5 ± 2.2	18.6 ± 1.2	20.0 ± 0.4	3.5 ± 0.6
18:2	1.7 ± 0.2	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.1	2.9 ± 0.5	1.0 ± 0.04	1.4 ± 0.3	0.2 ± 0.1
20:3n-9	2.1 ± 0.4	0.8 ± 0.3	6.5 ± 1.0	3.3 ± 0.7	1.5 ± 0.2	0.5 ± 0.04	1.1 ± 0.3	0.1 ± 0.05
20:4n-6	22.2 ± 0.6	8.0 ± 2.2	39.0 ± 1.9	9.6 ± 1.0	9.2 ± 0.6	1.9 ± 0.1	13.4 ± 1.6	1.2 ± 0.3
22:3n-9	0.7 ± 0.1	0.7 ± 0.1	4.9 ± 0.6	3.0 ± 0.5	0.7 ± 0.3	1.5 ± 0.1	4.0 ± 0.6	8.4 ± 0.3
22:4n-9	1.8 ± 0.3	1.0 ± 0.5	9.7 ± 0.9	7.5 ± 0.6	3.2 ± 0.2	8.6 ± 0.3	27.8 ± 0.2	52.9 ± 1.1
22:4n-6	1.9 ± 0.3	0.7 ± 0.2	5.1 ± 0.8	3.0 ± 0.2	0.7 ± 0.1	0.5 ± 0.1	1.5 ± 0.01	—[b]
22:5n-6	7.1 ± 1.1	19.8 ± 0.4	13.9 ± 1.5	55.0 ± 3.2	6.1 ± 1.3	23.5 ± 0.4	17.0 ± 2.2	27.7 ± 0.8
24:4 + 22:6	0.7 ± 0.4	0.9 ± 0.1	3.5 ± 0.3	3.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	1.0 ± 0.3	1.6 ± 0.2
24:5n-6	—[b]	0.4	—[b]	1.2 ± 0.1	—[b]	0.2 ± 0.1	—[b]	0.9 ± 0.1

[a] Results are given as percentages (wt%) of the fatty acids depicted (mean values ± SD from three and four individual samples, for epididymis and epididymal spermatozoa, respectively).

[b] Not detectable under the present GLC conditions (either too small for detection or not resolved from a closely eluting larger peak).

52.9 ± 2.3 and 17.8 ± 2.0%, and in the spermatozoa isolated from such region the same plasmalogens accounted for 42.1 ± 6.1 and 52.1 ± 4.1% of the total EGP and CGP, respectively. More than 60% of the fatty acids of plasmenylcholine, the major phospholipid of sperm from *cauda* epididymis, was accounted for by n-9 polyenes (Table 1).

DISCUSSION

The results presented here reveal some as yet uncharacterized features of the fatty acid composition of lipids of rat epididymis and epididymal spermatozoa. In addition to well-known polyenes such as 22:5n-6, mature rat spermatozoa are shown to be rich in uncommon long and very long polyenes of the n-9 series, the major one being 22:4n-9. The fact that rat sperm lipid classes are rich in polyenoic fatty acids is consistent with previous work in other animal species, such as the bull (15) and boar (16). However, whereas 22:5n-6 and 22:6n-3 are the major polyenes in the lipids of epididymal spermatozoa in these species, accounting for virtually all the acyl chains of the ether-linked choline and ethanolamine glycerophospholipids, the abundance of 22:4n-9 in plasmenylcholine appears to be a characteristic of rat sperm cells.

Mammalian spermatozoa are released from the testes with a generous supply of lipids rich in polyunsaturated fatty acids. During their journey through the epididymis, these cells undergo a series of maturational changes which include, among others, alterations in polyenoic fatty acids composition (Fig. 2). In the present study, polyenoic acids of the n-9 series of sperm were minor components of other tissues of rat, including testes, in agreement with what has long been known for testicular lipids, exceedingly rich in 22:5n-6 (17). Even taking into account that the epididymal tract is composed of many cell types, the amounts of 20:3n-9, 22:3n-9 and 22:4n-9 found in its glycerophospholipids were high in comparison with other rat tissues. The epididymis contains an active layer of androgen-dependent epithelial cells responsible for many of the molecular changes that lead to the functional maturity of spermatozoa. Such cells might also participate in metabolic activities resulting in the characteristic lipid pattern of mature spermatozoa, perhaps including the synthesis of 22:4n-9 and related fatty acids. It is interesting that for some lipids, like EGP, these fatty acids are more abundant in the epididymis than in sperm (Fig. 2 and Table 1).

The unusual polyenes of rat epididymis and sperm extend and fit into what is known about naturally occurring polyenoic fatty acids. The structural and metabolic relationship between n-9 polyenes and the more familiar and ubiquitous n-3 and n-6 polyenes are likely to be as summarized in Scheme 2. In liver, these conversion were

Enzymatic step	Oleate (n-9)	Linoleate (n-6)	Linolenate (n-3)	Position of first double bond from carboxyl end
	18:1n-9	18:2n-6	18:3n-3	9
Δ6 Desaturation	↓	↓	↓	
	18:2n-9	18:3n-6	18:4n-3	6
Elongation	↓	↓	↓	
	20:2n-9	20:3n-6	20:4n-3	8
Δ5 Desaturation	↓	↓	↓	
	20:3n-9	20:4n-6	20:5n-3	5
Elongation	↓	↓	↓	
	22:3n-9	22:4n-6	22:5n-3	7
Δ4 Desaturation	↓	↓	↓	
	22:4n-9	22:5n-6	22:6n-3	4
Elongation	↓	↓	↓	
	24:4n-9	24:5n-6	24:6n-3	6
	24:3n-9	24:4n-6	24:5n-3	9

SCHEME 2

studied in detail by Bernert and Sprecher (18). The presence of Δ9, Δ6 and Δ5 desaturases may account for all the major polyunsaturated fatty acids that occur in liver. A Δ8 desaturase activity, which is absent in liver (19), but has been demonstrated in rat testes (20), could lead to precursors of 22:4n-9, 22:5n-6 and 22:6n-3 through an alternative route, as shown in Scheme 3.

Enzymatic step	Oleate (n-9)	Linoleate (n-6)	Linolenate (n-3)
	18:1n-9	18:2n-6	18:3n-3
Elongation	↓	↓	↓
	20:1n-9	20:2n-6	20:3n-3
Δ8 Desaturation	↓	↓	↓
	20:2n-9	20:3n-6	20:4n-3

SCHEME 3

The most highly unsaturated members of each of the three series (namely 22:4n-9, 22:5n-6 and 22:6n-3) have in common that they are the products of an enzymatic activity that results in the presence of a double bond at C_4. Unsaturations are not known to occur in positions closer to the carboxyl end in naturally occurring long-chain fatty acids of mammalian membranes, and only 22-carbon fatty acids exhibit such a feature. The properties and regulation of this putative Δ4 desaturase have not yet been studied as thoroughly as those of the other desaturases. The vertebrate retina and the reproductive tract, rich in 22:6n-3, 22:5n-6 and 22:4n-9, seem to be the tissues of choice to investigate its activity.

Further elongation of 22-carbon polyenes to very long polyenoic fatty acids (C_{24} and longer) occurs in the reproductive tract (21) and in other tissues, such as retina (22). Polyenoic acids having 24-37 carbon atoms of the n-3 and n-6 series have been shown to occur in ejaculated spermatozoa of various mammals (23) and are predominantly associated with sphingomyelin (24). Similar very long polyenoic fatty acids have been observed in phosphatidylcholine from the retina of several vertebrates (12), including the rat (25). Given the presence of the unusually long 24:3n-9 and 24:4n-9 in rat spermatozoa observed in this study, longer polyenes of the n-9 series could be expected to be found in the future.

It is worth noting that long-chain n-9 polyenes do not occur in large proportions in sperm cells of species other than the rat (15,16,23). Among the explanations for the abundance of n-9 polyenes in rat spermatozoa in the presence of a normal supply of 18:2n-6 and 18:3n-3 in the diet is the possibility that the lipid metabolism in the rat epididymal tract is so demanding as to partially exhaust the n-6 and n-3 polyenoic precursors. This may result in a "local" deficiency of such polyenes within the epididymis, forcing the tissue to resort to the synthesis of such n-9 counterparts. In fact, the process of spermatogenesis in the rat is very active and continuous throughout the animal life. In addition to being non-renewable through synthesis *in situ*, essential fatty acids like 18:2n-6 could partly be unavailable if they are esterified in certain lipid classes or consumed in the synthesis of higher polyenes, such as 22:5n-6. The possibility of an important demand of essential fatty acids resulting in an extensive formation of n-9 polyenes in the rat still does not explain the remarkable selectivity observed in this study in the distribution of polyenes among major sperm lipid classes and subclasses. The finding of such an abundant natural source of long-chain n-9 polyenoic fatty acids as rat sperm apparently contradicts the widely accepted view that large amounts of these polyenes can only be observed in lipids of tissues from animals undergoing dietary deficiencies of fatty acids of the n-6 and n-3 series, showing that they can be normal, physiological components of certain cells.

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Isolation and Characterization of Novel 2-Hydroxy Fatty Acids from the Phospholipids of the Sponge *Smenospongia aurea*

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The Caribbean sponge *Smenospongia aurea* revealed the presence of six novel branched α-hydroxy fatty acids: 2-hydroxy-17-methyloctadecanoic acid, 2-hydroxy-21-methyldocosanoic acid, 2-hydroxy-22-methyltricosanoic acid, 2-hydroxy-22-methyltetracosanoic acid, 2-hydroxy-24-methylpentacosanoic acid, and 2-hydroxy-23-methylpentacosanoic acid. These novel α-hydroxy fatty acids were associated with phosphatidylethanolamine. The sponges *Aplysina lacunosa* and *Aplysina fistularis* also contained considerable amounts of α-hydroxy fatty acids, the very long-chain 5,9,23-tricontatrienoic acid (30:3), and phytanic acid. The sterol composition of the three sponges was also studied. It indicated that *A. lacunosa* and *A. fistularis* contained large amounts of aplysterol and verongulasterol, while *S. aurea* did not show any of these sterols. The results are discussed in terms of the taxonomy of the species.
Lipids 27, 681-685 (1992).

Sponges belonging to the genus *Smenospongia* and *Aplysina* have shown interesting morphological similarities that warrant a closer scrutiny of their secondary metabolites (1). The Caribbean sponge *Smenospongia aurea* (Hyatt, 1875) contains the 5-bromo- and 5,6-dibromo-*N,N*-dimethyltryptamines, aureol, and 6-bromoaplysinopsin as well as 6-bromo-4′-*N*-demethylaplysinopsin (1). The *Aplysina* species, identified as *Aplysina lacunosa* (Pallas, 1766) and *Aplysina archeri* (Higgin, 1875), were also shown to contain mixtures of these metabolites and/or bromotyrosine derived compounds. An earlier report indicated similar results for *S. aurea* and *Smenospongia (= Polyfibrospongia) echina* (2). It is of interest that the sponge previously known as *Spongia fenestra* or *Aplysina aurea* was reclassified as *Smenospongia aurea* (Hyatt) by Wiedenmayer (3).

Our research group studied the phospholipid fatty acid composition of the sponges *Verongula gigantea* and *Aplysina (= Verongia) archeri* and concluded that the similarity in their phospholipid fatty acid composition could be used as a chemotaxonomic tool (4). The key similarities in the phospholipids were the presence of predominantly saturated fatty acids, of considerable quantities of 3,7,11,15-tetramethylhexadecanoic (phytanic) acid and of the very long-chain 5,9,23-tricontatrienoic acid, and of considerable amounts of 2-hydroxy fatty acids. The sterol composition of these sponges was also similar, in particular because both sponges contained aplysterol, verongulasterol, and 25-dehydroaplysterol (5,6). However, the question still remains whether the Caribbean sponge *S. aurea* contains a lipid composition similar to other *Aplysina* sponges.

Literature published in this area prompted us to further study other sponges from the genus *Aplysina*, in particular *Aplysina lacunosa* and *Aplysina fistularis*, in search of similar lipid metabolites which could serve as a sound chemotaxonomic base. We also studied the lipids of the sponge *Smenospongia aurea* in order to compare these with the lipids of sponges of the family Aplysinidae. It was likely that *S. aurea* would contain α-hydroxy fatty acids. We also felt that this type of study could also lead to a better understanding of the metabolism of α-hydroxy fatty acids in sponges in the future.

EXPERIMENTAL PROCEDURES

Aplysina lacunosa was collected on May 25, 1989, at a depth of 24 m. *Aplysina fistularis* and *Smenospongia aurea* were collected March 22, 1991, at 12 m near the shelf edge of La Parguera, Puerto Rico. The sponges were placed in dry ice for transportation to the laboratory. They were freeze-dried on a Labconco Freeze Dryer 4.5 (Model 77510; Kansas City, MO). For extraction, approximately 180 g of dry sponge was carefully cleaned of all nonsponge debris and cut into small pieces. Extraction with 600 mL of chloroform/methanol (1:1, vol/vol) yielded total lipids. The neutral lipids, glycolipids and phospholipids (1.5 g) were separated by column chromatography on silica gel (60-200 mesh) using the procedure of Privett *et al.* (7). The sterols were obtained from the neutral lipids fraction by chromatography on a silica gel column using diethyl ether/hexane (1:1, vol/vol) as solvent. The sterol mixtures were recrystallized two times, the first time in MeOH, the second time in CH_3CN. Final separation was achieved using a Waters Associates high-performance liquid chromatography (HPLC) system (M510 pump; Waters 410 differential refractometer), with an Altex Ultrasphere ODS-2 column (25 cm × 4.6 mm i.d.) and methanol as solvent. The phospholipid classes were separated by preparative thin-layer chromatography (TLC) on silica gel G using chloroform/methanol/water (25:10:1, vol/vol/vol) as solvent. The fatty acid methyl esters from phospholipids were obtained by reaction with methanolic hydrogen chloride (8) followed by purification by column chromatography and elution with hexane/diethyl ether (9:1, vol/vol). The resulting methyl esters were analyzed by gas chromatography/mass spectrometry (GC/MS) using a Hewlett-Packard 5995A GC/MS (Hewlett-Packard, Palo Alto, CA) or a Hewlett-Packard 59970 MS ChemStation equipped with a 20 m × 0.32 mm nonpolar fused silica column (Supelco, Bellefonte, PA) with SPB™-1 as the bonded phase. Hydrogenations were carried out in 10 mL of absolute methanol in the presence of catalytic amounts of platinum oxide (PtO_2). ^1H NMR and ^{31}P NMR spectra were recorded on a General Electric GN-300 (300 MHz) spectrometer in $CDCl_3$. Mass spectral data for the key fatty acids studied are presented below.

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Abbreviations: ECL, equivalent chain length; EIMS, electron impact mass spectrometry; FTIR, Fourier transform infrared spectroscopy; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography.

2-Hydroxy-17-methyloctadecanoic acid methyl ester. MS (70 eV) *m/z* (rel. intensity), 328 (M^+, 3.7), 269 ($M^+ - 59$, 9), 159 (2.3), 151 (2.2), 149 (4), 145 (7), 141 (4), 139 (3), 137 (2.5), 132 (2), 129 (3.5), 127 (12), 125 (8.4), 123 (3.6), 113 (9), 111 (18), 109 (11), 103 (13.5), 99 (5.4), 97 (49), 96 (10), 95 (21), 90 (45), 87 (13), 74 (10), 71 (42), 69 (66), 67 (28), 59 (13), 57 (100), 55 (97).

2-Hydroxy-21-methyldocosanoic acid methyl ester. MS (70 eV) *m/z* (rel. intensity), 384 (M^+, 5.2), 325 ($M^+ - 59$, 5), 207 (2), 185 (2), 145 (5), 137 (2.5), 127 (11), 123 (6), 117 (11.5), 111 (21), 109 (15), 103 (11), 97 (48), 96 (16), 95 (27), 90 (36), 85 (23), 83 (53), 81 (29), 71 (57), 69 (60), 67 (29), 59 (12), 57 (100), 55 (99).

2-Hydroxy-22-methyltricosanoic acid methyl ester. MS (70 eV) *m/z* (rel. intensity), 398 (M^+, 5.8), 339 ($M^+ - 59$, 4.2), 207 (0.7), 170 (0.7), 159 (1.3), 156 (1.2), 151 (0.9), 145 (4.9), 141 (2.5), 137 (2.3), 127 (9.6), 125 (6.8), 117 (2.6), 113 (8.2), 111 (18), 109 (10), 103 (10.6), 99 (6.8), 98 (7), 97 (41), 95 (25), 90 (37), 85 (23), 83 (48.6), 81 (26), 75 (81), 71 (53), 69 (55), 68 (14), 67 (25), 57 (100), 55 (84).

2-Hydroxy-22-methyltetracosanoic acid methyl ester. MS (70 eV) *m/z* (rel. intensity), 412 (M^+, 7.5), 353 ($M^+ - 59$, 3.9), 354 (1.2), 207 (0.36), 169 (0.5), 159 (1.3), 156 (1.0), 145 (4.0), 143 (1.1), 141 (2.6), 137 (2.2), 127 (6.6), 125 (5.9), 123 (4.4), 117 (1.7), 113 (6.8), 111 (14.9), 109 (10), 103 (8.7), 99 (4.5), 97 (31), 95 (20.6), 90 (27.9), 87 (7.0), 85 (18.1), 83 (36.6), 82 (11.6), 81 (20.1), 75 (4.6), 74 (5.5), 71 (33.7), 69 (39.7), 67 (19.9), 57 (100), 55 (60).

2-Hydroxy-24-methylpentacosanoic acid methyl ester. MS (70 eV) *m/z* (rel. intensity), 426 (M^+, 5.8), 367 ($M^+ - 59$, 1.9), 207 (2.3), 159 (2.3), 145 (4.6), 143 (2.8), 141 (2.6), 137 (2.8), 127 (9.6), 125 (7.1), 123 (5), 117 (15), 113 (7.6), 111 (19.9), 109 (11), 103 (8), 99 (5.9), 97 (37), 95 (23.6), 90 (30.8), 87 (11.4), 85 (21.8), 83 (43.4), 82 (19.6), 81 (26.7), 75 (8.6), 74 (14.6), 71 (48.4), 69 (56.7), 67 (27.6), 57 (100), 55 (85).

2-Hydroxy-23-methylpentacosanoic acid methyl ester. MS (70 eV) *m/z* (rel. intensity), 426 (M^+, 5.1), 367 ($M^+ - 59$, 1.6), 159 (1.3), 152 (1.0), 145 (3.1), 141 (2.4), 137 (2.3), 127 (6.5), 125 (5.9), 123 (4.4), 117 (7.9), 113 (6.5), 111 (14.6), 109 (9), 103 (7.4), 97 (31.5), 95 (21.3), 91 (3.3), 90 (28), 87 (5), 85 (19.2), 83 (38.2), 82 (12.3), 81 (20.6), 75 (23), 71 (41), 69 (41), 67 (18), 61 (2.3), 59 (6), 57 (100), 55 (64).

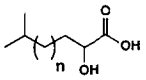
Lithium aluminum hydride reduction. The fatty acid methyl esters (5 mg) were placed in a 50-mL three-necked round-bottomed flask at −78°C in 20 mL of dry THF. Excess $LiAlH_4$ was added and the mixture was left to stir overnight. For the work-up, 10–15 mL of ethyl acetate followed by 10 mL of distilled water were added to destroy excess $LiAlH_4$. The solution was then acidified with H_2SO_4 followed by diethyl ether extraction (2 × 15 mL). The ether extract was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated to dryness, affording the corresponding 1,2-diols in almost quantitative yield.

Oxidative cleavage of the 1,2-diols. The 1,2-diols (2–3 mg) were dissolved in 2 mL of *t*-BuOH followed by the addition of 1 mL of a solution of $NaIO_4/KMnO_4$ in distilled water and 1 mL of K_2CO_3 in water. The mixture was stirred vigorously for 1 h, followed by acidification with conc. H_2SO_4. The excess oxidant was destroyed by adding $Na_2S_2O_3$ until the solution remained colorless. The organic extract was taken up with diethyl ether (3 × 4 mL), dried over anhydrous Na_2SO_4, and the solvent was evaporated to dryness affording the corresponding fatty acids.

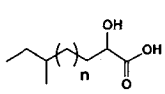
RESULTS

Phospholipid fatty acid compositions of *Smenospongia aurea, Aplysina lacunosa* and *Aplysina fistularis* are presented in Table 1. The main phospholipids in the sponges were phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylcholine (PC). PE was the principal phospholipid, while PC was a minor component. ^{31}P nuclear magnetic resonance (NMR) was used to analyze the phospholipid mixture as we did previously (4). The phospholipid fatty acid composition of these sponges represented a series of predominantly saturated fatty acids with considerable amounts of *anteiso* and *iso* acids. Phytanic (3,7,11,15-tetramethylhexadecanoic) acid was present in *ca.* 4% relative abundance in *S. aurea*, 9.4% abundance in *A. lacunosa*, and 9% abundance in *A. fistularis*. The mass spectrum of the phytanic acid methyl ester showed a base peak at *m/z* 101 (100%) and peaks at *m/z* 171 (15%) and *m/z* 241 (2%) indicating branching consistent with its structure (9). A second key fatty acid common to all three sponges was the very long chain 5,9,23-triacontatrienoic acid (30:3). This fatty acid was identified by its retention times in capillary gas chromatography (GC), equivalent chain length (ECL), and by mass spectral comparison with an authentic sample (10).

Each of the three sponges contained α-hydroxy fatty acids (Table 1). *A. lacunosa* contained a series of α-hydroxy acids (10% of the total fatty acid mixture) ranging in length from 15 to 23 carbon atoms, *A. fistularis* contained α-hydroxy acids from 19 to 24 carbon atoms (7% of the total fatty acid mixture), while *S. aurea* showed the most complete series of α-hydroxy acids ranging in length from 17 to 26 carbon atoms (29% of the total fatty acid mixture). These findings substantially extend current knowledge on α-hydroxy acids in sponges (4). In particular, *S. aurea* contained six novel branched α-hydroxy acids which were characterized as 2-hydroxy-17-methyloctadecanoic acid (1), 2-hydroxy-21-methyldocosanoic acid (2), 2-hydroxy-22-methyltricosanoic acid (3), 2-hydroxy-22-methyltetracosanoic acid (4), 2-hydroxy-24-methylpentacosanoic acid (5), and 2-hydroxy-23-methylpentacosanoic acid (6). All contained either *iso* or *anteiso* terminal methyl



(1) n= 13
(2) n= 17
(3) n= 18
(5) n= 20



(4) n = 18
(6) n = 19

branching. Characterization of these acids was by GC retention times, GC/MS, chemical degradation to the known saturated *iso*/*anteiso* saturated compounds, and nuclear magnetic resonance spectroscopy. A representative example is the characterization of 2-hydroxy-21-methyldocosanoic acid methyl ester, which showed a very characteristic MS spectrum with a molecular ion at *m/z* 384 (5%, $C_{24}H_{48}O_3$). The identification of an $M^+ - COOCH_3$ fragmentation ion at *m/z* 325 (5%) strongly

TABLE 1

The Phospholipid Fatty Acids and Aldehydes from the Sponges

	Abundance (%)		
	S. aurea	*A. lacunosa*	*A. fistularis*
Fatty acids			
Methyltridecanoic (14:0)	0.1	—	—
Tetradecanoic (14:0)	0.6	0.9	1.0
Methyltetradecanoic (15:0)[a]	8.0	5.1	14.2
Pentadecanoic (15:0)	1.0	1.5	1.0
Methylpentadecanoic (16:0)[a]	3.0	1.8	11.2
9-Hexadecenoic (16:1)	1.0	—	—
Hexadecanoic (16:0)	7.1	3.6	18.9
Heptadecenoic (17:1)	1.3	3.6	2.8
Methylhexadecanoic (17:0)[a]	12.0	6.9	8.9
Heptadecanoic (17:0)	0.6	1.5	0.8
Octadecenoic (18:1)	1.0	—	—
Methylheptadecanoic (18:0)[a]	0.4	—	—
Octadecanoic (18:0)	4.6	—	3.3
Methyloctadecanoic (19:0)[a]	9.2	—	—
Nonadecanoic (19:0)	0.5	7.0	5.7
3,7,11,15-Tetramethylhexadecanoic (20:0)	4.0	9.4	9.1
Eicosatetraenoic (20:4)	—	10.0	—
Eicosanoic (20:0)	2.4	2.7	1.3
Heneicosanoic (21:0)	0.5	0.9	—
Methyleicosanoic (21:0)[a]	0.2	—	—
Docosanoic (22:0)	1.7	1.5	—
Methyldocosanoic (23:0)[a]	0.8	—	—
Tricosanoic (23:0)	0.5	—	—
Methyltricosanoic (24:0)[a]	1.6	—	—
Tetracosanoic (24:0)	0.5	0.6	—
Methyltetracosanoic (25:0)[a]	3.7	—	—
Pentacosanoic (25:0)	1.1	—	—
5,9-Hexacosadienoic (26:2)	1.0	—	—
5,9,23-Tricontatrienoic (30:3)	1.6	8.2	9.4
α-Hydroxy acids			
2-Hydroxypentadecanoic (15h:0)	—	0.6	—
2-Hydroxy-14-methylpentadecanoic (*i*-16h:0)	—	0.3	—
2-Hydroxyhexadecanoic (16:0)	—	0.6	—
2-Hydroxy-15-methylhexadecanoic (*i*-17h:0)	0.1	0.3	—
2-Hydroxyheptadecanoic (17h:0)	0.3	0.6	—
2-Hydroxy-16-methylheptadecanoic (*i*-18:0)	1.0	—	—
2-Hydroxyoctadecanoic (18h:0)	1.4	1.2	—
2-Hydroxy-17-methyloctadecanoic (*i*-19h:0)[b]	0.4	—	—
2-Hydroxynonadecanoic (19h:0)	1.4	0.6	0.7
2-Hydroxyeicosanoic (20h:0)	5.5	—	2.6
2-Hydroxyheneicosanoic (21h:0)	1.3	1.5	0.9
2-Hydroxydocosanoic (22h:0)	2.1	1.8	1.1
2-Hydroxy-21-methyldocosanoic (*i*-23h:0)[b]	0.3	—	—
2-Hydroxytricosanoic (23h:0)	0.8	2.7	—
2-Hydroxy-22-methyltricosanoic (*i*-24h:0)[b]	1.7	—	—
2-Hydroxytetracosanoic (24h:0)	5.9	—	2.4
2-Hydroxy-23-methyltetracosanoic (*i*-25h:0)	2.1	—	—
2-Hydroxy-22-methyltetracosanoic (*ai*-25h:0)[b]	1.5	—	—
2-Hydroxypentacosanoic (25h:0)	1.6	—	—
2-Hydroxy-24-methylpentacosanoic (*i*-26h:0)[b]	0.3	—	—
2-Hydroxy-23-methylpentacosanoic (*ai*-26h:0)[b]	0.5	—	—
2-Hydroxyhexacosanoic (26h:0)	0.6	—	—
Aldehydes[c]			
Undecanal (11:0)	—	1.2	—
Tridecanal (13:0)	—	0.6	—
Pentadecanal (15:0)	—	0.9	—
Heptadecanal (17:0)	—	1.2	—
Octadecanal (18:0)	0.3	11.0	1.4
Nonadecanal (19:0)	—	1.8	—
Tetracosanal (24:0)	0.5	—	—
Pentacosanal (25:0)	—	2.1	—
Heptacosanal (27:0)	—	5.7	—

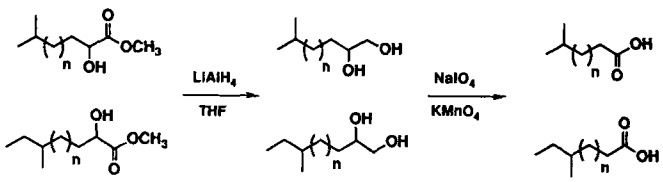
[a]Normally these were mixtures of *iso* and *anteiso* acids.

[b]First reported in a marine organism.

[c]Derived from alk-1-enylacyl phospholipids.

suggested α-substitution. Lack of significant peaks at *m/z* 74 and *m/z* 104, but the presence of fragmentation ions at *m/z* 90 (36%) and *m/z* 103 (11%) derived by McLafferty rearrangement, indicated the presence of the α-hydroxy structure. This was further confirmed by the long retention time in capillary GC with an ECL value of 23.88 found. The Fourier transform infrared (FTIR) spectrum of the mixture displayed a band between 3100 and 3600 cm^{-1}, supporting the presence of a hydroxy group. ^1H NMR of the 2-hydroxy fatty acid methyl esters showed the signal of the methoxy group of the ester at 3.78 ppm, while the signal of the methoxy group of a normal fatty acid methyl ester appears at 3.66 ppm. This downfield shift is consistent with α-hydroxy substitution. Moreover, the signal of the hydrogen at position 2 in the 2-hydroxy fatty acid methyl esters was observed between 4.1 and 4.2 ppm as expected for α-hydroxy fatty acid methyl esters. The identification of the entire series of long-chain α-hydroxy esters from *S. aurea* was possible based on a plot of retention time *vs.* number of carbon atoms. Three different families of α-hydroxy fatty acid methyl esters could thus be discerned.

The methyl branching in the novel α-hydroxy fatty acids was unequivocally determined by chemical degradation to the corresponding known *iso* and *anteiso* non-hydroxylated saturated fatty acids. The sequence used is shown in Scheme 1. The process resulted in a chain shortening



SCHEME 1

of the α-hydroxy esters by one carbon atom, while the non-hydroxylated saturated esters were unaffected. Hydrogenation of the mixture with PtO_2 in MeOH followed by $LiAlH_4$ reduction afforded the corresponding saturated 1,2-diols which were then cleaved and oxidized with $NaIO_4/KMnO_4$ to afford the corresponding nonhydroxylated fatty acid with one carbon less. These acids were then esterified with HCl/MeOH in order to obtain the corresponding fatty acid methyl esters. In all cases, the fatty acid methyl esters thus obtained corresponded to the saturated *iso* or *anteiso* methyl esters with one carbon less as shown by their ECL values as well as by GC coinjection with authentic standards. For example, the 2-hydroxy-22- and 23-methyltetracosanoic acid methyl esters (*ai*-25h:0 and *i*-25h:0) were transformed into the corresponding 23- and 24-methyltricosanoic acid methyl esters (*ai*-24:0 and *i*-24:0) by the sequence of reactions outlined above. The former methyl ester showed an ECL value of 23.63 and the latter an ECL value of 23.72. Similar results were obtained for the 2-hydroxy-23- and 24-methylpentacosanoic acid methyl esters (*ai*-26h:0 and *i*-26h:0) which were also transformed into the corresponding 22- and 23-methyltetracosanoic acid methyl esters (*ai*-25:0 and *i*-25:0) with ECL values of 24.75 and 24.63, respectively. By means of a series of recrystallizations from CH_3CN, we were able to isolate the 2-hydroxytetracosanoic acid methyl ester from *A. fistularis,* thus permitting the measurement of the optical rotation in methanol which was found to be a small positive number ($[\alpha]_D = +4.0°$). Therefore, α-hydroxy fatty acids from sponges seem to belong to the D-series.

A series of saturated aldehydes was also characterized which arose from 1-*O*-alk-1-enyl-2-acyl glycerophospholipids, commonly known as plasmalogens (11). While *S. aurea* produced only two aldehydes, namely octadecanal (18:0) and tetracosanal (24:0), *A. lacunosa* produced a more complete series of aldehydes ranging in length from 11 to 27 carbon atoms and accounting for 25% of the total long-chain components of the phospholipids of this sponge. Heptacosanal (27:0) is the longest aldehyde yet derived from sponge plasmalogens (11). The aldehydes were characterized as their dimethyl acetals. Dimethyl acetals normally do not afford a molecular ion in electron impact mass spectrometry (EIMS), but an ion equivalent to $[M - 31]^+$ (loss of a methoxy group) permits determination of the molecular weight. As an example, heptacosanal dimethyl acetal displayed a $[M - 31]^+$ peak at *m/z* 409 (0.5% abundance) and a base peak at *m/z* 75 ($C_3H_7O_2^+$).

The sterols from the three sponges were also isolated, using reverse-phase high-performance liquid chromatography (HPLC) for the final separation. The particular sterols were characterized by 300 MHz ^1H NMR, MS, and by comparison with authentic samples. The *Aplysina* sponges contained, as their major sterol 24*S*,26-dimethyl-25*S*-cholest-5-en-3β-ol (12). From *A. fistularis,* the following sterols were isolated and characterized: cholesterol (12% of the total sterol mixture), 24-methylcholesta-5,22-dien-3β-ol (5%), 24-methylcholest-5-en-3β-ol (5%), 24-ethylcholesta-5,22-dien-3β-ol (3%), 24-ethylcholest-5-en-3β-ol (29%), (24*R*,25*S*)-24,25-dimethylcholest-5-en-3β-ol (39%), and (24*R*)-24,26,27-trimethylcholesta-5,25-dien-3β-ol (6%). Similar results were obtained for *A. lacunosa* where 24*S*,26-dimethyl-25*S*-cholest-5-en-3β-ol (aplysterol) accounted for 39% of the total sterol mixture. On the other hand, in *S. aurea* we could detect neither aplysterol nor verongulasterol. The sterol mixture of *S. aurea* consisted of cholesterol (61% of the total sterol mixture), 24-methylcholesta-5,22-dien-3β-ol (10%), 24-methyl-cholest-5-en-3β-ol (5%), 24-ethylcholesta-5,22-dien-3β-ol (2%), and 24-ethylcholest-5-en-3β-ol (22%).

DISCUSSION

We observed that sponge phospholipid can contain α-hydroxy fatty acids ranging in length from 15 to 26 carbons. We also showed that sponges, such as *S. aurea,* are able to synthesize unusual very long chain branched α-hydroxy fatty acids and introduce them into their phospholipids. A series of *iso* and *anteiso* α-hydroxy fatty acids from 14 to 17 carbon atoms in length were previously identified in the phosphatidylethanolamines from *Nocardia leishmanii* (13); the 2-position of PE was exclusively substituted by branched α-hydroxy acids, while the 1-position contained chiefly *iso*-C_{16}, *iso*-C_{17}, and *anteiso*-C_{17} acids. A similar pattern was earlier reported for the lipids of *Streptomyces sioyaensis,* where 2-hydroxy-13-methyltetradecanoic acid was the main α-hydroxy acid, which was also found in the β-position in PE (14). A more

recent report showed that 2-hydroxy fatty acids, such as 2-hydroxy-15-methylhexadecanoic acid, are also found in PE from myxobacteria. Essentially two types of PE were encountered: one contained the nonhydroxy fatty acids, while the other contained 50% hydroxy fatty acids next to nonhydroxy fatty acids (15). *S. aurea* showed a similar picture except that *S. aurea* can synthesize longer chain fatty acids.

A second conclusion from our work relates to the chemotaxonomy of the sponges studied here. The phospholipid fatty acid compositions of *A. lacunosa* and *A. fistularis* were found to be quite similar to the ones reported for other sponges belonging to the family of the Aplysinidae, *i.e., Aplysina archeri* and *Verongula gigantea* (4). *S. aurea* also possesses a very similar fatty acid composition, except that more saturated *iso* and *anteiso* fatty acids were found in this sponge. The most interesting finding, however, was the difference in sterol composition between the *Smenospongia* and *Aplysina*. In both *A. lacunosa* and *A. fistularis* we found aplysterol to be the main sterol of both sponges (39% of the total fatty acid composition) and verongulasterol to be a minor constituent. We previously had obtained similar results for *A. archeri* and *V. gigantea* (5,6). *S. aurea*, on the other hand, showed a common sponge sterol composition, and no traces of aplysterol and/or verongulasterol were detected. Therefore on the basis of their sterol composition, the *Smenospongia* and *Aplysina* represent a different genus.

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A Facile Synthesis of 4-Hydroxy-2(*E*)-nonenal

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A facile, efficient synthesis of 4-hydroxy-2(*E*)-nonenal is presented as an alternative to the approaches published previously, which either employed four to six separate steps or furnished low yields. The commercially available 3(*Z*)-nonenol was sequentially oxidized into 3,4-epoxynonanol by 3-chloroperoxybenzoic acid followed by oxidation of the alcohol by periodinane to afford 4-hydroxy-2(*E*)-nonenal by this two-step procedure in 48 ± 7% yield.
***Lipids* 27, 686–689 (1992).**

4-Hydroxy-2(*E*)-nonenal (4HNE), a product of lipid peroxidation, is well-known for its biological effects, which include genotoxicity and cytotoxicity. Recently, Esterbauer *et al.* (1) have published a comprehensive review concerning 4HNE and related aldehydes.

A number of synthetic strategies for 4HNE and its homologs have been developed. Previous procedures had utilized variations of the Grignard reaction (2–6), isomerization of 2-yne-1,4-diols with a ruthenium catalyst (7), a method *via* 1,3-*bis*(methylthio)allyl-lithium (8) and a sequence of addition/elimination/substitution reactions performed on the corresponding saturated aldehyde (9,10). The present communication describes a facile two-step synthesis of 4HNE starting from 3(*Z*)-nonenol.

MATERIALS AND METHODS

Materials. 3(*Z*)-Nonenol (95%), 3-chloroperoxybenzoic acid (80–85%), hexanal (99%), 2-nonanone (99+%), pyridinium chlorochromate (98%), 2-iodobenzoic acid (98%), *O*-benzylhydroxylamine hydrochloride and potassium bromate were from Aldrich Chemical Company (Milwaukee, WI); $CHCl_3$ (high-performance liquid chromatography [HPLC] grade, 99.9%) was from Mallinckrodt (Paris, KY). All other chemicals and solvents were either reagent or HPLC grade. Thin-layer chromatography (TLC) plates were Silica Gel 60 F-254 (20 × 20 × 0.25 cm) from Merck (Darmstadt, Germany).

*Synthesis of 4-hydroxy-2(*E*)-nonenal.* To a solution of 3 (*Z*)-nonenol (0.5 mmol) in 2 mL $CHCl_3$ was added a 1.2 molar excess of 80–85% 3-chloroperoxybenzoic acid (130 mg). The solution was kept at room temperature for 1 h after which 2 mL of 10% $NaHCO_3$ was added with vigorous stirring for 45 min. As expected, the water washed $CHCl_3$ layer contained 3,4-epoxynonanol as determined by ^1H NMR and ^{13}C NMR spectrometry. The ^1H NMR data are given as chemical shift in ppm (δ) downfield from tetramethylsilane (TMS) and (number of protons, multiplicity, coupling constant in Hz and carbon assignment) are as follows: 3.84 (3H, *m* and brs, C-1 and probably OH); 3.09 (1H, apparent overlapping *dd* and *dd*, *J* values determined by simulation $J_{2a,3}$ = 4.4, $J_{2b,3}$ = 8.4, $J_{3,4}$ = 4.4, C-3); 2.95 (1H, apparent overlapping *dd* and *dd*, *J* values determined by simulation $J_{3,4}$ = 4.4, $J_{4,5a}$ = 5.7, $J_{4,5b}$ = 6, C-4); 1.87 and 1.67 (2H, *m*, C-2); 1.51 (4H, *m*, C-5,6); 1.32 (4H, *m*, C-7,8); 0.87 (3H, *t*, C-9). The ^{13}C NMR data are given relative to TMS in chemical shift in ppm (δ) and carbon assignment: 60.6, C-1; 56.9, C-4; 55.1, C-3; 31.6, C-7; 30.5, C-2; 27.8, C-5; 26.1, C-6; 22.5, C-8; 13.9, C-9. The assignments were confirmed by two-dimensional proton-carbon correlation spectroscopy, correlative spectroscopy (COSY) and distortionless enhancement by polarization transfer (DEPT) experiments (data not shown).

Subsequently, the alcohol group of 3,4-epoxynonanol was oxidized to the corresponding aldehyde by the reagent, periodinane. Periodinane was prepared by $KBrO_3$-H_2SO_4 oxidation of 2-iodobenzoic acid into the cyclic tautomer of 2-iodoxybenzoic acid followed by acetylation to the triacetate as described (11). In actual practice, the washed $CHCl_3$ layer containing 3,4-epoxynonanol from the previous step was dewatered for direct oxidation by periodinane in $CHCl_3$ without any other clean-up. Typically, the $CHCl_3$ layer was percolated through a 0.5 i.d. × 6 cm column (Pasteur pipette) packed with anhydrous Na_2SO_4, and the epoxynonanol was washed from the column with $CHCl_3$ to give a total volume of 5 mL (other dewatering procedures can be used). The $CHCl_3$ solution containing 3,4-epoxynonanol was oxidized by a molar excess of periodinane based on the starting 0.5 mmol 3(*Z*)-nonenol (generally a 2 molar excess, 1 mmol). An excess of periodinane is recommended because the reagent is sensitive to moisture (11). Various batches of periodinane could be checked for oxidation potential by reacting with an alcohol, such as 3(*Z*)-nonenol, and assessing conversion to the aldehyde by GLC. Also, it should be noted that certain suppliers of $CHCl_3$ add to their formulation as much as 1% ethanol as a preservative, in which case the alcohol must be removed before use. After the oxidation mixture was stirred for 20 min at room temperature, 14 mL of diethyl ether and 4 mL of 1.3 M NaOH were added, and the mixture was vigorously agitated for about 15 s. The aqueous NaOH layer containing most of the spent periodinane was removed, and an additional 5 mL of 1.3 m NaOH were added with vigorous stirring or vortexing for 10 min. The NaOH layer was removed, and the organic phase was washed with 5 mL of H_2O. Evaporation of solvent afforded crude 4HNE which gave the following ^1H NMR data in chemical shift, ppm (δ), and (number of protons, multiplicity, coupling constant in Hz, and carbon assignments): 9.56 (1H, *d*, *J* = 7.9, C-1); 6.29 (1H, *ddd*, *J* = 7.9, 15.7, 1.6, C-2); 6.81 (1H, *dd*, *J* = 15.7, 4.7, C-3); 4.42 (1H, *dtd*, *J* = 6.5, 4.7, 1.6, C-4). Except for minor differences, the ^1H NMR spectrum was in agreement with previously published data (12). The Fourier transform-infrared (FT-IR) spectrum gave the following diagnostic absorptions (cm^{-1}): 3438 m, br, OH; 2933 s, CH; 2863 m, CH; 2732 w, aldehyde CH; 1690 s, carbonyl; 1127 and 1077 m, hydroxyl CO; 976 s, *E*-olefin. An electron impact-mass spectrometry (EI-MS) furnished the following

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Abbreviations: COSY, correlative spectroscopy; DEPT, distortionless enhancement by polarization transfer; EI-MS, electron impact-mass spectrometry; FT-IR, Fourier transform-infrared; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; 4HNE, 4-hydroxy-2(*E*)-nonenal; HPLC, high-performance liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane; UV, ultraviolet.

major ions [*m/z* (% relative intensity, ion structure)]: 127 (3, M^+-CHO); 109 (7, M^+-CHO-H_2O); 99 (9); 86 (13); 85 (12); 71 (12); 57 (78); 43 (100); 41 (45).

Formation of O-benzyloximes of 4HNE. Yields of 4HNE were determined by gravimetric analyses of the *O*-benzyloximes of 4HNE synthesized with *O*-benzylhydroxylamine reagent (4 molar excess of *O*-benzylhydroxylamine based on the reactant, 3*Z*-nonenol). The *O*-benzylhydroxylamine reagent was prepared by a method previously described for *O*-pentafluorobenzylhydroxylamine reagent (13). The *syn* and *anti* oximes were extracted from the reagent by $CHCl_3$ and isolated by TLC as described below. Trimethylsilyloxy derivatives were prepared for gas chromatography/mass spectrometry (GC/MS) by reaction with chlorotrimethylsilane/hexamethyldisilazane/pyridine (3:2:2, vol/vol/vol).

Chromatographic analysis. The purity of 4HNE was assessed by TLC using two different solvents: Solvent A, $CHCl_3/CH_3OH$ (99:1, vol/vol) and Solvent B, hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol); 4HNE R_f 0.14–0.15 and 0.33, respectively. Detection was by sequential viewing of 4HNE by ultraviolet (UV) absorption, followed by visualization of an orange spot after spraying with 0.4% 2,4-dinitrophenylhydrazine in 2N HCl. Other nonaldehydic components were visualized by spraying with vanillin/ethanol/H_2SO_4 (160:40:1, wt/vol/vol) and charring. 3(*Z*)-Nonenol and 3,4-epoxynonanol were analyzed by TLC using hexane/diethyl ether (3:2, vol/vol) as developing solvent [3(*Z*)-nonenol R_f 0.37; 3,4-epoxynonanol R_f 0.12]. *O*-Benzyloximes of 4HNE (*syn* and *anti*) were isolated by preparative TLC using hexane/ethyl acetate (4:1, vol/vol). Just prior to TLC, the excess *O*-benzylhydroxylamine was reacted with hexanal to avoid close migration of the hydroxylamine with the 4HNE oximes. The *syn* and *anti* oximes were detected by UV absorbance of two closely migrating bands centered at about R_f 0.30, and the TLC scrapings were eluted with ethyl acetate.

Gas-liquid chromatography (GLC) was completed with a Spectra-Physics (San Jose, CA) Model SP-7100 gas chromatograph equipped with an open tubular capillary column (12 m × 0.22 mm) coated with SE 30. The carrier gas flow was about 1 mL/min, and the temperature was programmed from 100–140°C at 5°C/min. 3(*Z*)-Nonenol, 3,4-epoxynonanol, and 4HNE eluted after 1.8, 3.5 and 3.9 min, respectively.

Open-column liquid chromatography was completed with a 1 cm i.d. × 10 cm column packed with SilicAR CC-4 (Mallinckrodt) slurried in hexane. Components of the product mixture were eluted with 60 mL each of 5, 10, 20 and 30% diethyl ether in hexane. HPLC was accomplished with a 9.4 × 250 mm Zorbax Sil 5μ spherical particle column (Dupont Instruments, Wilmington, DE) using hexane/acetone (87:13, vol/vol) elution at 1.5 mL/min and UV-detection at 218 nm.

Spectral analyses. ^1H and ^{13}C NMR spectra were obtained in C^2HCl_3 solution with a Bruker Model WM-300 spectrometer. Electron ionization-mass spectrometry (EI-MS) was done on a Hewlett-Packard (Palo Alto, CA) Model HP 5970 mass-selective detector equipped with a coupled gas chromatograph. The gas chromatograph was equipped with a fused silica capillary column (15 m × 0.25 mm) coated with a bonded nonpolar DB-1 (J&W Scientific, Rancho Cordova, CA). For elution of product mixtures containing 4HNE, the column temperature was held at 50°C for 4 min, and then programmed at 10°/min using a carrier gas flow of about 0.5 mL/min. A temperature program from 120°C–200°C at 10°C/min was employed to elute the trimethylsilyloxy derivative of 4HNE-*O*-benzyloxime. Infrared (IR) spectra were obtained from a liquid film sandwiched between KBr plates using a Galaxy 6020 series FT-IR spectrometer from Mattson Instruments (Madison, WI). IR absorptions are described in terms of wave number (cm^{-1}), intensity and assignment, in that order, using the following abbreviations: w, weak; m, medium; s, strong; and br, broad.

RESULTS

The synthetic method employed first epoxidation of 3(*Z*)-nonenol by 3-chloroperoxybenzoic acid followed by oxidation of the alcohol to the aldehyde by periodinane (theoretically affording 3,4-epoxynonanal). However, epoxyaldehydes, analogous to 3,4-epoxynonanal, are known to be readily converted to the corresponding hydroxy-2-ene aldehydes by conditions as mild as silica gel in diethyl ether (14) or triethylamine in diethyl ether (15), and accordingly the 3,4-epoxynonanal conversion to 4HNE would be expected to proceed by mild treatment with either acid or base (16). As discussed below, it seems certain that the NaOH wash ostensibly used to remove spent periodinane (2- iodobenzoic acid) was responsible for the conversion of 3,4-epoxynonanal to 4HNE (Fig. 1). 4HNE was synthesized by this simple two-step procedure without change of the $CHCl_3$ solvent and without chromatographic purification. As illustrated by the two-dimensional proton-carbon correlation nuclear magnetic resonance (NMR) spectrum (Fig. 2), there was little evidence for significant impurities in the crude product. Carbon assignments were confirmed by DEPT. Chromatographic purification by either preparative TLC, open-column liquid chromatography (elution volume 140–180 mL) or, preferably, HPLC (retention time, 35 min) is recommended to eliminate minor impurities. As indicated by GC-MS, impurities probably were methyl 2-iodobenzoate, nonadienal and 3-oxononanal (data not shown); however, the latter two could have been partially or wholly produced by the GLC conditions.

Ordinarily, it was most convenient to assess the product yield by GLC of the underivatized product mixture, which afforded an estimate of 60% yields. However, due to the possibility of artifact formation on the GLC column and lack of response factors, these uncertainties prompted us to determine the yield of the reaction by gravimetric determination of the *O*-benzyloxime derivative of 4HNE which afforded a calculated overall yield of 48 ± 7% (n = 7).

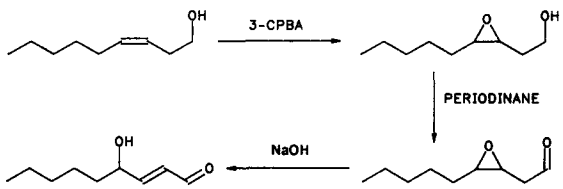

FIG. 1. Synthetic route to 4-hydroxy-2(*E*)-nonenal from 3(*Z*)-nonenol; 3-CPBA, 3-chloroperoxybenzoic acid.

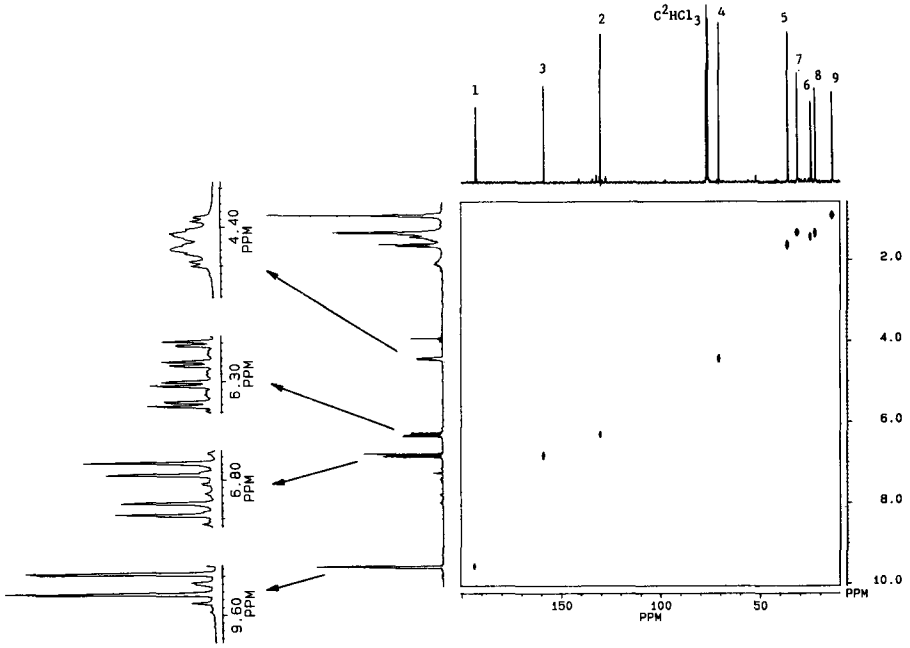

FIG. 2. Two-dimensional proton-carbon correlation NMR of crude synthetic 4-hydroxy-2(*E*)-nonenal (no chromatographic purification).

The *O*-benzyloxime of 4HNE was determined to be authentic by GC-MS of the trimethylsilyloxy derivative which afforded very similar spectra for both *syn* and *anti* isomers. The last eluting and most abundant isomer gave the following ions [*m/z* (% relative intensity, ion structure)]: 333 (3, M^+); 262 (15, $M^+ - CH_3(CH_2)_4$); 242 (42, $M^+ - CH_2C_6H_5$); 226 (14, $M^+ - OCH_2C_6H_5$); 91 (100, $CH_2C_6H_5$); 73 (47, trimethylsilyl).

As briefly mentioned above, we were able to observe a compound consistent in properties with the hypothetical intermediate, 3,4-epoxynonanal. When the organic layer containing the periodinane oxidation products was briefly treated with NaOH for 15 s and then washed with water instead of further agitating with NaOH for 10 min, a compound comprising about 60% of the total GLC peak area was detected at a retention of 2.4 min, compared to a 3.9 min retention time for 4HNE. Further agitation with NaOH for 10 min resulted in disappearance of the peak at retention of 2.4 min with a corresponding increase in the 4HNE peak. Furthermore, this intermediate was detected by TLC as a 2,4-dinitrophenylhydrazine reactive spot, which did not absorb UV, and migrated at a somewhat larger R_f value than 4HNE. GC-MS of this compound suggested that the intermediate was in fact 3,4-epoxynonanal giving the following major ions [*m/z* (% relative intensity, ion structure)]: 138 (0.1, $M^+ - H_2O$); 127 (2.6, $M^+ - CHO$); 113 (2.4, $M^+ - CH_2CHO$); 99 (22); 85 (5, $M^+ - (CH_2)_4CH_3$); 83 (37); 71 (25); 57 (56); 55 (100, $CH{=}CHCHO$); 43 (53, CH_2CHO); 41 (81).

Periodinane oxidation of the alcohol, 3,4-epoxynonanol, to 4HNE was determined to be somewhat superior to another oxidation agent of alcohols, pyridinium chlorochromate (17). GLC of the products of pyridinium chlorochromate oxidation indicated that the yield of 4HNE was only about 30% of the total peaks detected. Furthermore, a relatively large GLC peak detected at 2.4 min retention was indicative of 3,4-epoxynonanal. A 10 min NaOH treatment of the mixture dissolved in organic solvent, as described above for the periodinane oxidation, led to disappearance of the 2.4 min peak with a corresponding increase in the 4HNE peak. Although NaOH treatment apparently improved the yield of 4HNE, other unidentified GLC peaks were also observed even after alkali treatment. Possibly, the relatively long time (1 h) used for oxidation leads to epoxide opening and further oxidation. That 4HNE was produced by pyridinium chlorochromate oxidation was proved by TLC of the product mixture by sequentially using solvents B and A and obtaining a ^1H NMR spectrum of the isolate. Despite its apparent drawbacks, the pyridinium chlorochromate method does provide an alternative to periodinane oxidation if one can tolerate the increased impurities and loss in yield. With some effort expended on varying the conditions of pyridinium chlorochromate oxidation, it may be possible to further improve the yields.

A more direct route to 4HNE would appear to be direct epoxidation of 3(*Z*)-nonenal; however, aldehydes, such as 3(*Z*)-nonenal, are sensitive to the common epoxidizing reagent, 3-chloroperoxybenzoic acid. For example, reaction of hexanal with this reagent under the conditions used here largely oxidized the aldehyde into hexanoic acid (data not shown).

DISCUSSION

Previous methods for synthesis of 4HNE and its homologs usually employed variations of the Grignard reaction as a synthetic strategy (2-6). Although these methods required four to five steps, the overall yields were generally good, in the 50-78% range (2-5). A procedure offered an alternative to 4HNE in six steps employing conversion of nonanal by a series of addition, elimination

and substitution reactions (9,10), and 4-hydroxyhexenal has been prepared *via* 1,3-*bis*(methylthio)allyl-lithium and propanal in 61–62% yield (8). A potentially promising one-step procedure used to convert dodec-2-yne-1,4-diol into 4-hydroxy-2(*E*)-dodecenal by a ruthenium catalyst in 44% yield would appear to be limited by the availability of non-2-yne-1,4-diol (7). The two-step synthesis reported here does not require a change of solvent, only provided that the $CHCl_3$ solvent be reasonably dried after the epoxidation step prior to reaction with periodinane. Due to the relatively clean conversion, chromatographic purification was straightforward.

The current synthesis of 4HNE was designed to mimic a proposed biosynthetic pathway by plants. Recently, it was shown that a soybean preparation containing hydroperoxide lyase activity mainly converted 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid into 9-oxononanoic acid and 4HNE (18). Although a good yield of 3(*Z*)-nonenal was anticipated from the hydroperoxide lyase activity, this compound was only a minor product. This observation implied that the soybean preparation was converting 3(*Z*)-nonenal into 4HNE. One hypothesis is that a hydroperoxide-dependent peroxygenase or epoxygenase was epoxidizing 3(*Z*)-nonenal followed by conversion into 4HNE. Such enzymes from soybean (19) and *Vicia faba* (20) are known to epoxidize unsaturated fatty acids using as oxidants lipid hydroperoxide or H_2O_2. Recently, it was shown that 13*S*-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid and the alcohol, 3(*Z*)-nonenol, supplied to the soybean preparation produced 3,4-epoxynonanol (Gardner, H.W., unpublished). Accordingly, it seemed plausible that the peroxygenase could be responsible for the formation of 4HNE from 3(*Z*)-nonenal *via* 3,4-epoxynonanal. This seemed to be an especially attractive possibility in light of the hypothesis of Pryor and Porter (16) that conversion of 3,4-epoxynonanal may be one of the origins of 4HNE during peroxidation of lipids. In fact, their hypothesis was the key to suggesting such a biosynthetic pathway to 4HNE and the current method of synthesis. Experiments with *Vicia faba* hydroperoxide-dependent epoxygenase indicated that the conversion of 3(*Z*)-nonenal to 4HNE involves two separate reactions, one of which was shown to be catalyzed by the hydroperoxide-dependent epoxygenase (Gardner, H.W., and Hamberg, M., communicated at the 8th International Conference on Prostaglandins and Related Compounds, Montreal, Canada, July 26–31, 1992).

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Specificity of Soybean Lipoxygenase-1 in Hydrated Reverse Micelles of Sodium *bis*(2-Ethylhexyl)sulfosuccinate (Aerosol OT)

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Soybean lipoxygenase-1 (EC 1.13.11.33) was purified by fast protein liquid chromatography on a MONO Q column and studied with respect to the conversion of linoleic and arachidonic acids in reverse micelles of sodium *bis*(2-ethylhexyl)sulfosuccinate in *n*-octane. In this system the specific activities were lower by one order of magnitude than those in the corresponding aqueous system. High-performance liquid chromatography analyses indicated the predominant formation of 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-HpODE) from linoleic acid and of 15*S*-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15) from arachidonic acid both in the aqueous system and in the reverse micelles. After sedimentation of the hydrated reverse micelles by ultracentrifugation, both linoleic acid and 13-HpODE were found to be enriched in the micelles with only small amounts of these compounds present in *n*-octane. It is proposed that substrates and products of the lipoxygenase reaction are located mainly in the surfactant shell of the hydrated reverse micelles and reach the micelle-entrapped enzyme by diffusion into the aqueous interior space.
Lipids 27, 690–693 (1992).

Reverse micelles are formed when suitable amphiphilic molecules such as AOT [sodium *bis*(2-ethylhexyl)sulfosuccinate] are dissolved in nonpolar organic solvents; they differ from normal micelles in that the polar head groups are oriented toward the interior of the spheroidal aggregates. In reverse micelles, water and other polar compounds, including enzymes, can be encapsulated. The physical chemistry of hydrated reverse micelles of AOT in *n*-octane has been studied in detail by several research groups (1,2).

Several authors (3–6) have shown that commercially available soybean lipoxygenase-1 (EC 1.13.11.33) of low activity oxygenates linoleic acid in reverse micelles. The reaction products have not been identified, a step that is required to characterize the specificity of the reaction. In the aqueous system, soybean lipoxygenase-1 oxygenates polyenoic fatty acids containing an n-8 doubly allylic methylene to their corresponding n-6 hydroperoxy derivatives (for details see ref. 7). In this case the methyl end of the substrate appears to be a signal for the site of initial hydrogen abstraction. Under special conditions, such as change in pH or absence of doubly allylic methylene in the corresponding position, an inverse binding of the substrate at the active site of the enzyme is also possible (7). Thus, soybean lipoxygenase-1, which is an arachidonate "15-lipoxygenase", converts 15*S*-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HpETE) to 5*S*,15*S*-dihydroperoxy-6*E*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (8).

In an earlier study with reverse micelles of AOT, it was proposed that linoleic acid is incorporated into the micelle with the carboxylic group being directed toward the aqueous cavity containing the enzyme, and that only the substrate intercalated in the boundary surfactant layer would be transformed by the lipoxygenase (4). Such an assumption would imply that the carboxylic group, rather than the methyl tail, of the fatty acid molecule is accessible for the enzyme. In this case the lipoxygenase should act as "5-lipoxygenase" forming 5-HpETE and 9-HpODE from arachidonic acid and linoleic acid, respectively. The aim of the present study was to clarify whether the regio- and stereospecificities of highly active soybean lipoxygenase-1 in hydrated reverse micelles of AOT are identical to those in an aqueous system or whether inverse binding of the substrate occurs at the active site of the enzyme.

MATERIALS AND METHODS

Chemicals. AOT (Aerosol OT; dioctylsulfosuccinate, sodium salt; sodium *bis*(2-ethylhexyl)sulfosuccinate; research grade) was purchased from Serva (Heidelberg, Germany). *n*-Octane was purified by shaking three times with 0.03–0.04 volumes of 20% sulfur trioxide in concentrated sulfuric acid (oleum), followed by repeated washes with concentrated sulfuric acid, water, 2% NaOH, and again with water until neutral. After drying over anhydrous sodium sulfate, the solvent was distilled by means of a Vigreux column and shown to be spectrally pure in the ultraviolet (UV) region. Linoleic acid (analytical grade) and arachidonic acid (research grade) were obtained from Serva. Since the linoleic acid contained detectable amounts of oxygenation products, as judged from both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), it was purified on a silica gel column with *n*-hexane/diethyl ether (9:1, vol/vol) as developing solvent.

Purification of lipoxygenase. Fifty grams of air-dried vegetable soybeans, variant *Dorado* (VEB Saat- und Pflanzgut, Quedlinburg, Germany) were homogenized by means of a rotating knife-homogenizer (coffee grinder). The soya flour was defatted three times at −18 to −21°C (ice/NaCl mixture) with 100 mL acetone each. The defatted flour (about 42 g) was extracted with 7.5 vol of oxygen-free sodium acetate buffer, pH 4.5, for 1 h under shaking. The extract was centrifuged [Sorvall angle centrifuge (Newton, CT) at 16,000 × *g*, 15 min, 4°C] and the pellet was discarded. The lipoxygenase was precipitated by ammonium sulfate at a saturation of 0.63 at 0°C. The precipitate was dissolved in 0.01 M Tris buffer (pH 6.8) and dialyzed against the same buffer and frozen in small portions in liquid nitrogen until use.

Final purification was performed at 22°C by fast protein liquid chromatography using an analytical MONO Q HR5/5 column; (Pharmacia-LKB, Freiburg, Germany);

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Abbreviations: AOT, Aerosol OT, dioctylsulfosuccinate sodium salt, sodium *bis*(2-ethylhexyl)sulfosuccinate; 13-HpODE, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; 15-HpETE, 15*S*-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; DNP, 2,4-dinitrophenol; MOPS, 4-morpholino-propanesulfonic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography, UV, ultraviolet.

16 mg protein of the ammonium sulfate precipitate were applied. 4-Morpholino propanesulfonic acid (MOPS) buffer (0.01 M), pH 6.8, was used as starting solution, elution was by a gradient of NaCl (up to 0.2 M) in the same buffer. The buffers were degassed before use by sonication. Soybean lipoxygenase-1 was eluted at 0.18 M NaCl and shown to be 94% pure by polyacrylamide gel electrophoresis in sodium dodecylsulfate using a 10–20% gradient gel and Coomassie blue staining. Its turnover number immediately after purification at 25°C with linoleic acid as substrate was 350 s^{-1}. The preparation was devoid of lipoxygenase-2 and -3 as judged by the lack of aerobic formation of oxodienoic acids absorbing at 285 nm in water (9). For comparison, a commercial preparation of soybean lipoxygenase (Serva) was used which exhibited a turnover number of only 12 s^{-1}. 15-Lipoxygenase from rabbit reticulocytes was prepared as described (10).

Lipoxygenase assay. The activity of soybean lipoxygenase-1 in the aqueous system was measured at 25°C either spectrophotometrically at 234 nm (most experiments) or oxygraphically (experiment in Fig. 1) in 0.1 M borate buffer pH 9.0 with either linoleic acid or arachidonic acid as substrate. The substrate solutions were prepared by mixing 0.53 M stock solution in methanol with an equal volume of 0.53 M KOH and addition to 50 vol of borate buffer. The clear substrate solution was added to the assay mixture to a final concentration of 200 μM.

For activity measurements in the reverse micellar system at 25°C, the substrate fatty acid was dissolved in 1 mL of 0.05 M AOT in *n*-octane. Unless stated otherwise, a final concentration of 1.25 mM linoleic acid was used. Thereafter, 20 μL of 0.1 M sodium borate buffer, pH 9.0, was added and the mixture was vigorously shaken until optically transparent. After a preincubation period of 2 min, the reaction was started by addition of 2 μL of enzyme solution (final concentration, 20 nM). These conditions correspond to a hydration degree (molar ratio of water/AOT; w_0 value) of 25, which has been reported to afford maximal lipoxygenase activity (4,5). The reaction was followed by means of a Shimadzu UV-2100 spectrophotometer (Duisburg, Germany) at 245 nm. Measurements at the absorbance maximum of the conjugated dienes were not possible, owing to the high absorbance of AOT at this wavelength.

The oxygenation rates were estimated from the absorbance changes using molar absorption coefficients of 23,000 $M^{-1}cm^{-1}$ at 234 nm and 14,500 $M^{-1}cm^{-1}$ at 245 nm, respectively. The molecular activities of the lipoxygenase were calculated from the oxygenation rates and the protein concentrations in the assay samples presuming a molecular mass of 100,000 for soybean lipoxygenase-1.

Analytical methods. The reaction products from linoleic acid or arachidonic acid were identified by straight-phase HPLC using a Shimadzu instrument coupled to a Hewlett-Packard diode array detector 1040 A (Bad Homburg, Germany) on a Zorbax-SIL column (250 × 4.6 mm, 5 μm particle size; DuPont, Wilmington, DE) with the solvent system *n*-hexane/isopropanol/acetic acid (100:2:0.1, vol/vol/vol), and a flow rate of 2 mL/min. The absorbance at 235 nm (conjugated dienes) was recorded. For identification of the enantiomers, the methyl esters of the hydroxypolyenoic fatty acids (obtained by reduction of the hydroperoxy compounds by triphenylphosphine and subsequent methylation with diazomethane) were separated by chiral-phase HPLC on a dinitrobenzoyl phenylglycine column (chiral phase ionicly linked to silica gel, 260 × 4.6 mm, 5 μM particle size, Baker Chemical Co., Gross-Gerau, Germany) with the solvent *n*-hexane/isopropanol (100:0.25, vol/vol) at a flow rate of 1 mL/min (11). The compounds were generally identified by co-injection of authentic standards which were prepared and purified by HPLC and identified as described earlier (11).

The concentrations of linoleic acid and 13-HpODE (13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid) were determined by reverse-phase HPLC on a Nucleosil C18 column (KS system, 250 × 4 mm, 5 μm particle size; Macherey-Nagel, Düren, Germany) with the solvent methanol/water/acetic acid (10:10:0.1, vol/vol/vol) at a flow rate of 1 mL/min; linoleic acid and 13-HpODE were detected at 205 nm and 235 nm, respectively. The chromatograms were quantified by integration of peak areas.

Sedimentation of the reverse micelles. A 5-mL sample of the lipoxygenase assay mixture in reverse micelles of AOT (see above) was centrifuged at 180,000 × *g* for 2.5 h at 25°C in a Beckman L8-70 ultracentrifuge (Palo Alto, CA; swing-out rotor) before or 20 min after addition of lipoxygenase. In the latter case, oxygen gas was bubbled through the mixture before ultracentrifugation to avoid anaerobic conditions owing to the continuation of the lipoxygenase reaction. Moreover, 0.21 mM 2,4-dinitrophenol (DNP) as a marker for aqueous reverse micelles was added to the samples before ultracentrifugation. After ultracentrifugation, fractions of 0.5 mL were collected by means of a syringe and analyzed for DNP (optical density at 345 nm) and for linoleic acid and 13-HpODE (reverse-phase HPLC, see analytical methods).

RESULTS

In agreement with other authors (3–6) we found that soybean lipoxygenase-1 is active in hydrated reverse micelles of AOT. The reaction toward linoleic acid or arachidonic acid was linear for 15–20 min (not shown), indicating that neither depletion of substrate fatty acid, nor of oxygen, nor time-dependent inactivation of enzyme occurred under these conditions. The molecular activities of the enzyme in the aqueous and in the organic system containing reverse micelles are listed in Table 1. It is evident that in the aqueous system both the highly active purified enzyme and the commercially available preparation are more active than in the organic system by one order of magnitude.

One possible reason for the comparatively low activities in hydrated reverse micelles might be the inhibition of the lipoxygenase by AOT, which was shown to occur in the aqueous system; 50% inhibition was observed at about 1.5 mM AOT (Fig. 1). In this experiment, the lipoxygenase activity was assayed oxygraphically because of the turbidity that occurred in the aqueous system at high concentrations of AOT. The inhibition did not depend on the concentration of linoleic acid in the range between 0.16 and 1.0 mM. This observation argues against a competition between substrate and AOT for the active site of the enzyme. For this reason, the inhibition by AOT cannot account for the $Ks_{0.5}$ values in the reverse micellar system which have been reported to be two orders of magnitude higher than in the aqueous system (4,5).

TABLE 1

Rates of Dioxygenation of Polyenoic Fatty Acids by Soybean Lipoxygenase-1 in Aqueous and Organic Systems at 25°C

	Linoleic acid		Arachidonic acid
	Own preparation	Serva	Serva
Medium	$V_m(s^{-1})$	$V_m(s^{-1})$	$V_m(s^{-1})$
0.1 M Sodium borate buffer, pH 9.0	100[a]	12	18
Reverse micelles of 0.05 M AOT in *n*-octane (w_0 = 24)	6.8	3.3	5.3

[a]Actual value after storage of the purified enzyme for five months.

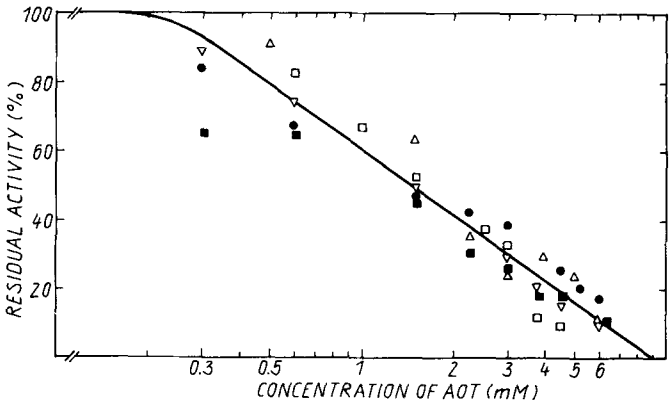

FIG. 1. Inhibition of soybean lipoxygenase-1 by AOT in 0.1 M borate buffer, pH 9.0, using commercial enzyme (Serva). Lipoxygenase activity was measured oxygraphically with linoleic acid as substrate at concentrations of 0.16 mM (□), 0.20 mM (△), 0.32 mM (▽), 0.48 mM (●), and 1.0 mM (■).

We failed to observe a reaction of the 15-lipoxygenase from rabbit reticulocytes in hydrated reverse AOT micelles under various experimental conditions (not shown). In the aqueous system we observed complete inhibition of this enzyme at AOT concentrations as low as 0.1 mM. We conclude, therefore, that the reticulocyte 15-lipoxygenase may be completely inhibited by AOT in the organic micellar system.

The reaction products of soybean lipoxygenase-1 from linoleic and arachidonic acids in both the aqueous system and the organic system containing reverse micelles were identified by straight-phase high-pressure liquid chromatography (Fig. 2 and Table 2). In both systems, products turned out to be nearly exclusively 13-HpODE and 15-HpETE as judged by co-injection of authentic standards. Separate experiments using chiral-phase HPLC indicated that in all cases (both with the commercial enzyme and the highly active enzyme) the *S* enantiomer was formed exclusively (Table 2). Thus, the specificity of soybean lipoxygenase-1 in hydrated reverse AOT micelles is actually identical to that in aqueous systems.

To estimate the distribution of substrate and product in the *n*-octane/H_2O/AOT system, the hydrated reverse micelles were sedimented by ultracentrifugation. DNP was added as a marker for the micelles, since this compound is known to be exclusively entrapped in the reverse micelles (12). After ultracentrifugation, fractions were collected and analyzed for DNP, linoleic acid and 13-HpODE. The data are compiled in Table 3. It is evident that both linoleic acid and 13-HpODE are strongly associated with the reverse micelles (bottom fractions 8 and 9). The two compounds appear to differ, however, with respect to their concentration in the *n*-octane phase. While 13-HpODE

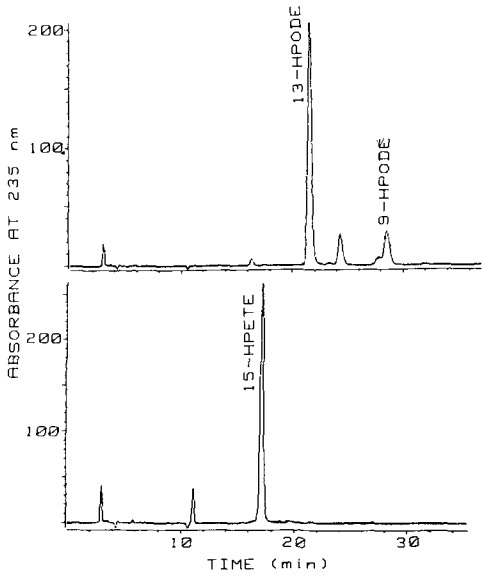

FIG. 2. Straight-phase HPLC of the dioxygenation products from linoleic (upper panel) and arachidonic acid (lower panel) in hydrated reverse micelles of AOT in *n*-octane. Purified soybean lipoxygenase-1 (20 nM) was incubated with the substrate fatty acid (see Materials and Methods) for 20 min at 25°C. Aliquots were directly applied to the HPLC column.

TABLE 2

Dioxygenation Products from Linoleic and Arachidonic Acids in an Aqueous System and in an Organic System Containing Reverse Micelles of AOT[a] (w_0 = 23)

	Products (%) from		
	Linoleic acid		Arachidonic acid
Medium	13-HpODE	9-HpODE	15-HpETE
0.1 M Sodium borate buffer (pH 9.0)	95 (96:4 *S/R*)[b]	5 (50:50 *S/R*)	>98 (99% *S*)
Reverse micelles of AOT in *n*-octane	>98 (99:1 *S/R*)	—	>98 (>99% *S*)

[a]Soybean lipoxygenase-1 (50 nM in the aqueous system, 200 nM in the organic system) was incubated at 25°C with the substrate fatty acid for either 5 min in the aqueous system or 20 min in the organic system. The reaction products were extracted from the aqueous system twice with diethyl ether and analyzed by straight-phase HPLC. From the organic system, aliquots were directly applied onto the HPLC column.

[b]The percentages of the *S* and *R* enantiomers are given in parentheses. For analysis, the hydroperoxy fatty acids were reduced with triphenylphosphine followed by methylation of the carboxylic group with diazomethane. The hydroxypolyenoic acid methyl esters obtained were repurified by straight-phase HPLC and thereafter separated by chiral-phase HPLC (see Materials and Methods).

TABLE 3

Distribution of 2,4-Dinitrophenol, Linoleic Acid and 13*S*-Hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-HpODE) in Reverse Micelles of AOT (w_o = 23) After Ultracentrifugation

Fraction no.	DNP (rel. conc.)[b]	Linoleic acid[a] (rel. conc.)[b]	13-HpODE (rel. conc.)[b]
1 (top)	—	0.078	0.015
2	0.020	0.098	0.021
3	0.036	0.098	0.018
4	0.040	0.106	0.031
5	0.065	0.12	0.033
6	0.075	0.16	0.045
7	0.093	0.20	0.054
8	0.55	0.73	0.83
9 (bottom)	1.0	1.0	1.0

[a]The distribution of linoleic acid in a sample without enzyme was similar (not shown).

[b]The concentration of the bottom fraction was set as unity; absolute values: DNP, A_{345} = 8.40; linoleic acid, 123 mg/mL; and 13-HpODE, 0.67 mg/mL.

largely follows the sedimentation course of DNP indicating nearly exclusive localization in the micelles, linoleic acid exhibits about four-fold higher relative concentrations in the upper fractions containing only small amounts of reverse micelles, which suggests a low but detectable concentration of linoleic acid in *n*-octane.

The experiment described in Table 3 does not discriminate between the possibilities of a preferred localization in the AOT monolayer or in the aqueous interior space. However, in a separate experiment we found that in the two-phase system *n*-octane/0.1 borate buffer (pH 9.0) containing AOT, almost the entire amount of linoleic acid is located, after phase separation, in a milky interphase, whereas only small amounts were present in both the *n*-octane and the aqueous phases (not shown). This observation argues against a preferential localization of linoleic acid in the aqueous interior of the micelles.

DISCUSSION

The data presented here indicate that the specificity of the reaction of soybean lipoxygenase-1 in hydrated reverse micelles of AOT is identical to that in aqueous systems. The data further show that the substrate fatty acid is preferentially incorporated into the micelles rather than dissolved in *n*-octane. It is reasonable to assume that the polyenoic fatty acid is preferably localized in the AOT monolayer (surfactant shell) of the reverse micelles, as is known for naphthoic acid, which is also a nonpolar carboxylic acid (2). Considering their solubility properties, a preferred localization of fatty acids in the aqueous interior of the AOT micelles appears rather unlikely. The results of the analysis of lipoxygenase products in the reverse micelles are in line with the assumption that the fatty acid reaches the active site of the enzyme by diffusion from the surfactant shell into the aqueous interior. The alternate possibility that the enzyme directly reacts with the fatty acid intercalated in the surfactant shell can be excluded. In the latter case, the carboxylic group of the fatty acid would be primarily accessible for the enzyme, which would lead to the formation of 9-HpODE and 5-HpETE, respectively.

The much lower activity of soybean lipoxygenase-1 in hydrated reverse micelles of AOT than in water differs from the behavior of a variety of other enzymes showing superactivity in the organic system (13,14). The reason for this peculiarity of lipoxygenases is not clear. Both inhibition of lipoxygenases by AOT and limited substrate diffusion to the active site of the enzyme may play a role. A dose-dependent inhibition of soybean lipoxygenase-1 by AOT was demonstrated in the aqueous micellar system. It is, however difficult to extrapolate from these conditions to those that exist in hydrated reverse micelles. Moreover, one cannot extrapolate the lipoxygenase activity in reverse micelles of AOT to conditions without inhibition by AOT.

Despite the disadvantage of low activity, the reaction of soybean lipoxygenase-1 in hydrated reverse micelles of AOT may be useful for the preparation of certain hydroperoxy fatty acids. During our study we observed that the product from linoleic acid, namely 13-HpODE, was stable for several days after the end of the reaction in reverse micelles, whereas in the aqueous system there is a rapid destruction of the hydroperoxy compound unless special precautions are taken.

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Cell Membranes and Multilamellar Vesicles: Influence of pH on Solvent Induced Damage

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Pigment leakage from sheep and horse erythrocytes and from red beet tissue induced by non-polar solvents was determined as a function of pH. The results were compared to disruption of multilamellar vesicles (MLV) composed of phospholipids with equimolar cholesterol under identical conditions of solvent exposure and pH. Solvent access to cholesterol was used to measure vesicle disruption. MLV were made from 1,2-dioleoyl phosphatidylethanolamine, sphingomyelin (SP) and various phosphatidylcholines to simulate the major lipid components of membranes. Pigment leakage from erythrocytes caused by petroleum hydrocarbon (b.p. 60–80°C) was maximal at pH 2–4 and at pH 10, but minimal at pH 6.8; alcohols caused less pigment leakage than petroleum hydrocarbon. Betacyanin leakage from beet tissue induced by petroleum hydrocarbon was maximal at pH 2, with very little leakage at pH 4, 6.6 and pH 10. Alcohols caused minimal damage to beet tissue above pH 2. Cholesterol removal by petroleum hydrocarbon from MLV of mixed lipid composition was maximal at pH 2–4, reduced at pH 6.8 and minimal at pH 10. Lipid mixtures in which fatty acyl side chains of one phospholipid were of a different length than the other lost more sterol than mixtures in which the acyl side chains were of identical chain length. MLV with more than 25% SP lost more sterol than those with less or no SP. Results show that in mixtures of phospholipids, SP exposes the hydrocarbon phase of a bilayer to solvent extraction, a property that was also observed in native membranes. Erythrocyte membranes, which contain SP, were more severely damaged by petroleum hydrocarbon than beet cells, which have none. Membranes from erythrocytes were more prone to solvent disruption at pH 10 than MLV, but they were more resistant at physiological pH. It is suggested that conformational changes in membrane proteins due to shifts in pH cause exposure of hydrophobic portions of surrounding lipids to the environment. At neutral pH, the native conformation of proteins is expected to stabilize the bilayer of membranes.

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We have applied a simple physico-chemical procedure to study the stability of bilayers formed from single or mixed phospholipids. The criterion for stability was the capacity of bilayers to retain cholesterol upon exposure to a non-polar solvent. The assumption was that if phospholipids form stable bilayers, the polar head groups would repel the solvent and allow for only limited extractability of cholesterol. If the packing of the bilayer was unstable, or if the lipids formed a reverse hexagonal phase, cholesterol would become accessible to the non-polar solvent and the sterol would be extracted. This method was used previously to study the influence of side chain mobility, head group type and pH on the stability of bilayers (1,2). Results showed that at moderately acid to neutral pH, phospholipids with mobile side chains and/or those with ethanolamine-containing head groups form unstable bilayers from which cholesterol and phospholipids are readily removable, which may be a manifestation of reverse hexagonal phase formation (3). Conditions which would cause the head groups to occupy more space, *i.e.,* like charges and increased hydration, would increase the stability of bilayers.

In the present study, we compared the effect of organic solvents upon pigment leakage from whole cells to disruption of the lipid structure of multilamellar vesicles (MLV) caused by the same solvents. Leakage of betacyanin from beet root cells and of hemoglobin from erythrocytes were used as indicators of disruption of cell membranes. Pigment leakage was compared to cholesterol extraction from mixed lipid MLV under similar conditions of pH and solvent exposure. Cell membranes were expected to differ from model systems because native membranes include substantial amounts of protein which may be subject to conformational changes by pH and because the lipid compositions of model vesicles were not exact duplicates of those in membranes.

MATERIALS AND METHODS

Fresh beets were purchased from a local market. Sheep and horse erythrocytes were obtained from Environmental Diagnostics (Burlington, NC). Blood cells were washed at least three times with isotonic phosphate buffer, pH 6.8, prior to use and were made up to a uniform 50% suspension in the same buffer. Cells were held at 4°C until use.

Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL); bovine brain sphingomyelin (SP) was obtained from P-L Biochemicals (Milwaukee, WI); dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was from Sigma Chemical Co. (St. Louis, MO). Cholesterol was recrystallized twice from methanol. [4-^{14}C]Cholesterol was obtained from Amersham (Arlington Heights, IL). The purity of phospholipids was checked by thin-layer chromatography on silica gel G plates developed with chloroform/methanol/water (65:25:4, vol/vol/vol) and visualized with iodine vapors. All other substances and solvents were reagent grade and were used without further purification.

Stability of cell membranes. The stability of beet root membranes was determined by a modification of the method described by Grunwald (4). Cores were cut from beets with a cork borer (#4) and sectioned freehand with a razor blade into 3-mm slices. The end slices from each core were discarded and the remaining slices were washed under cold running tap water for several hours or overnight. To initiate an experiment, 10 root slices were placed

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Abbreviations: CAPS, cyclohexylaminopropanesulfonic acid; C/M, chloroform/methanol 2:1 (vol/vol); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; H_{II}, reverse hexagonal phase; MES, 2-(*N*-morpholino)ethanesulfonic acid; MLV, multilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SP, sphingomyelin; TLC, thin-layer chromatography.

in each of fifteen 100-mL beakers, with 9.0 or 10.0 mL of a designated sucrose-buffer solution. Buffers were 0.4 molal with respect to sucrose. The buffers used were 0.1 M KH_2PO_4 and phosphoric acid at pH 2.0; 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES)/NaOH at pH 4.0; KH_2PO_4/NaOH at pH 6.6 or 6.8; 0.1 M cyclohexylaminopropanesulfonic acid (CAPS)/NaOH at pH 10.0. For controls, beet root slices were incubated in 10.0 mL of a sucrose-buffer; for solvent treated samples, they were incubated in 9.0 mL of a sucrose-buffer with 1.0 mL of either methanol, ethanol, a 2:1 (vol/vol) mixture of chloroform/methanol, or petroleum hydrocarbon (b.p. 60–80°C). The organic solvents were added at the start of an experiment. At time zero and periodically thereafter, the absorbance of betacyanin at 535 nm was determined. All incubates were kept at 22–23°C in a constant temperature bath with continuous shaking. Incubations continued for 2 h, and all experiments were run in duplicate.

For experiments on erythrocytes, incubations were carried out at room temperature. The same series of buffers described for the beet cell experiments were used except that pH 6.8 was substituted for pH 6.6 and the buffers were made isotonic with NaCl. Experiments were initiated by addition of 200 μL of a 50% red cell suspension. The contents of each tube were mixed immediately after addition of red cells and were agitated by vortexing briefly every 20 s. After 10 min, the tubes were centrifuged for 10 or 20 min in a benchtop centrifuge to sediment unlysed cells, membranes and denatured hemoglobin. Absorbance of supernatants was read at 590 nm. All experiments were run in triplicate.

Stability of multilamellar vesicles. The effect of pH on pigment leakage from cells or vesicles was compared to the loss of cholesterol from bilayers prepared from pure lipids. MLV were prepared from phospholipids in approximately similar proportions to those reported in the literature for that type of cell. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and SP lipids with equimolar amounts of sterol, to which [4-^{14}C]cholesterol (22,000 cpm) had been added, were incorporated into MLV. The procedure for making and incubating MLV and extracting them with petroleum hydrocarbon has been described previously (2). A slight change in the procedure was made in that lipid films were suspended in 0.4 mL of appropriate buffer solutions for 10 min prior to vortexing with glass beads, and the samples were left undisturbed for 45 to 60 min prior to extraction with solvent. In some of the experiments, the three sequential petroleum hydrocarbon extracts of each sample were assayed individually. Extracted cholesterol was determined by scintillation counting of the residue left, after evaporation of the solvent, in Ecoscint fluid (National Diagnostics, Manville, NJ). The pattern of cholesterol removal from the vesicles revealed information about the environment of the hydrocarbon components of the bilayers.

RESULTS

Betacyanin leakage from beet root cells. Incubation of beet root slices in sucrose-buffer solutions with and without organic solvents for a period of 2 h showed that near maximum amounts of pigment leakage due to chloroform/methanol (C/M) occurred within 30 min after the start of the experiment. Data for pH 6.6 are shown in Figure 1. Pigment leakage with other solvents increased linearly over the 2 h time span with leakage caused by solvents only slightly higher than that observed in samples with no solvent (Fig. 1, insert). In the beet cell and later in the erythrocyte experiments, we observed that pigment leaking from cells was affected by pH. Hemoglobin turned brown and precipitated at low pH, while betacyanin slowly turned yellow at high pH. Denatured hemoglobin exhibited maximum absorbance at 590 nm. Betacyanin was measured immediately at the end of incubation. Alterations in the pigments due to pH were reflected in changes in absorbance. In order to compare samples at one pH with the others, we assigned pigment leakage due to C/M as the maximum (100%) that could be obtained from cells at any given pH. Pigment leakage from all other samples, including controls with no organic solvent, were compared to C/M samples at the same pH and expressed as percent of maximum leakage. We calculated the percentage of maximum damage based upon pigment leakage after 30 min of incubation. The experiment was repeated twice and yielded the same pattern of results each time, but absolute values for pigment leakage differed with different batches of beets.

Results for one experiment are depicted in Figure 2. At pH 2.0, the samples without organic solvent showed considerable pigment leakage. Addition of any organic solvent increased the amount of betacyanin leakage, but ethanol and petroleum hydrocarbon caused the most damage. At pH 4.0 and 6.6, none of the samples with or without organic solvent showed any significant amount

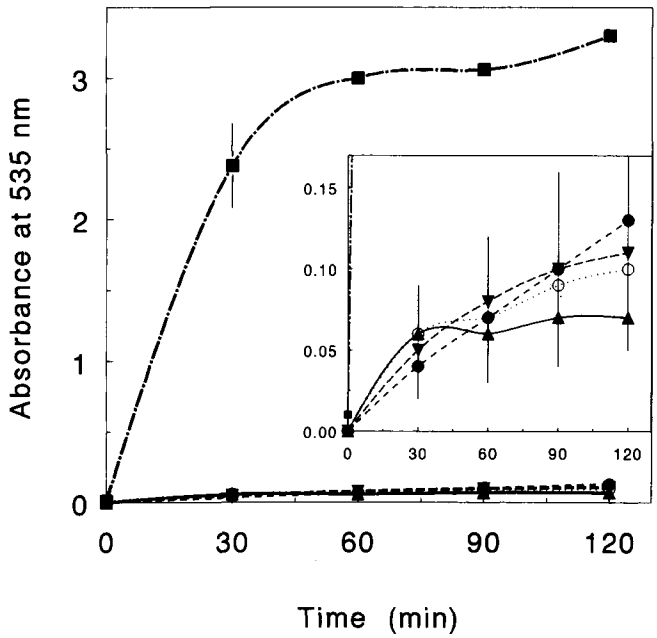

FIG. 1. Effect of solvents on betacyanin leakage from beet root tissue at pH 6.6 as function of time. Pigment leakage was measured by the increase in absorbance at 535 nm of the sucrose-phosphate buffer. Organic solvents were present to the extent of 10% of the volume of the suspending medium. The insert shows data on an expanded ordinate. All points are averages for three independent experiments. Standard deviations are within symbols or are shown by vertical bars. ▲, Buffer alone; ○, methanol; ▼, ethanol; ●, petroleum hydrocarbon; ■, chloroform/methanol (2:1, vol/vol).

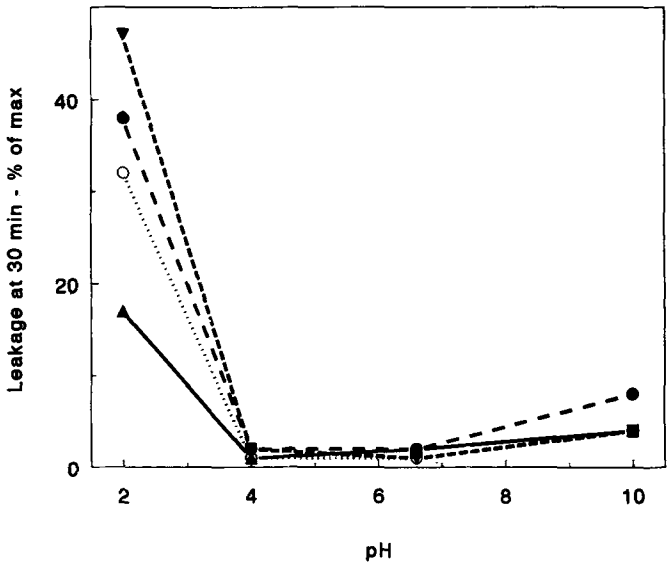

FIG. 2. Effect of solvents on betacyanin leakage from beet root tissue as function of pH at 30 min. Absorbance at 535 nm of samples exposed to an organic solvent at a given pH was compared to absorbance at the same pH and wavelength of samples exposed to chloroform/methanol (2:1, vol/vol). Organic solvents were present to the extent of 10% the volume of the suspending medium. Conditions are described under Materials and Methods. All points are averages for three independent experiments. Standard deviations are included within symbols or are shown by vertical bars. ▲, Buffer alone; ○, methanol; ▼, ethanol; ●, petroleum hydrocarbon.

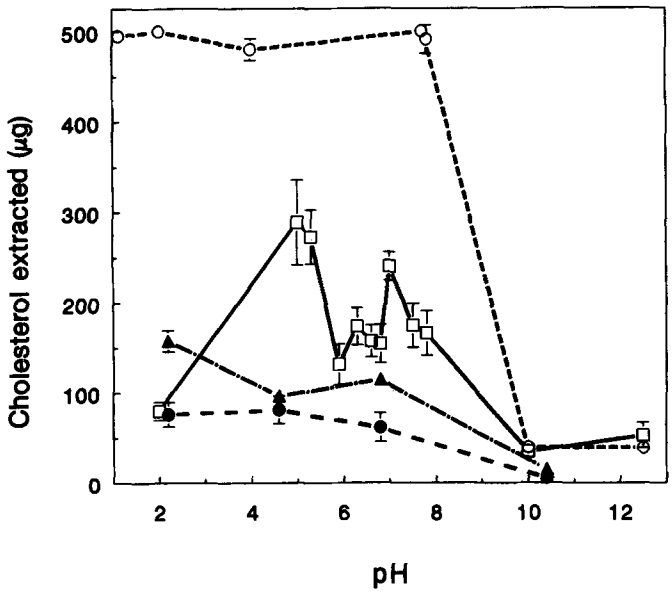

FIG. 3. Cholesterol extraction from MLV of DOPC/cholesterol (1:1, molar ratio), DOPE/cholesterol (1:1, molar ratio), DOPC/DOPE/cholesterol (1:1:2, molar ratio) or SP/DOPC/DOPE/cholesterol (0.5:0.5:1:2, molar ratio) related to the pH of the suspension media. MLV were prepared from pure phospholipids with [4-^{14}C]cholesterol equimolar to total phospholipids. The MLV were extracted three times with petroleum hydrocarbon. Details are given in the Methods section. Plots show the sum of cholesterol removed in all extracts. Experiments were done in triplicate and averages are indicated. Standard deviations are included in the symbols or are shown by vertical bars. □, DOPC; ○, DOPE; ▲, DOPE/DOPC; ●, SP/DOPC/DOPE.

of pigment leakage compared with leakage caused by C/M. At pH 10.0, pigment leakage from samples without organic solvent increased slightly, but organic solvents caused little, if any, additional leakage. Except at pH 2.0, organic solvents did not significantly aggravate damage due to changes in pH.

In order to compare the behavior of beet cells with that of pure lipid bilayers, MLV of lipids similar to those found in beet root tissue were prepared. Phospholipid analysis of beet root tissue reported by Beiss (5) showed that PC constituted 35% of the lipid and PE 17%, with phosphatidylserine (PS) and phosphatidic acid making up the balance. A combination of DOPC/DOPE/cholesterol (1:1:2, molar ratio) was used as an analog to beet cell membranes. We have previously found the extractability characteristics of MLV made from PC with two 18:1 side chains (DOPC) to be very similar to those of PC with one 16:0 and one 18:1 side chain (2). These MLV lost decreasing amounts of cholesterol upon extraction as the pH increased. At pH 2.2, about 150 μg or 30% of the sterol were lost; at pH 4.6 and 6.8, 100 μg or 20% was lost and at pH 10.4, less than 50 μg or 10% was lost (Fig. 3). While this pattern of data was similar to that observed with whole cells, a minimum of cholesterol extraction did not occur in the physiological range, nor were the properties of the mixed lipid vesicles an intermediate between the characteristics of the single lipid components. To determine whether similarity of head groups or of acyl side chains was responsible for the characteristics of the lipid vesicles, MLV of DPPC and DOPE (1:1) with equimolar cholesterol were prepared and studied for cholesterol loss to nonpolar solvent. Results are shown in Figure 4. These mixed lipid vesicles had much the same characteristics with respect

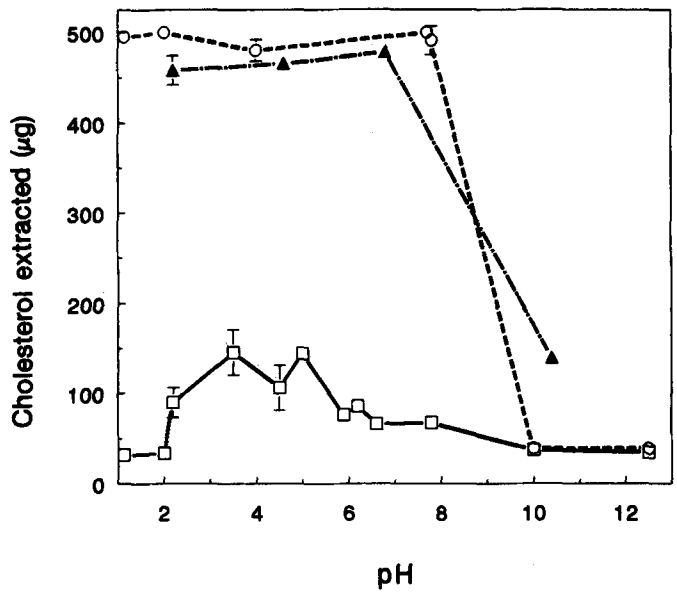

FIG. 4. Cholesterol extraction from MLV of DPPC/cholesterol (1:1, molar ratio), DOPE/cholesterol (1:1, molar ratio) and DPPC/DOPE/cholesterol (1:1:2 molar ratio) as function of pH of suspending media. Legend as in Figure 3. □, DPPC; ○, DOPE; ▲, DPPC/DOPE.

to cholesterol extraction as those prepared from DOPE alone. More than 450 μg or 90% of the cholesterol was extracted at pH values below 6.8. At pH 10.4, 150 μg or 30% of the cholesterol was extracted, but this was more than

the amount extracted from single species vesicles. Data from these experiments and those reported earlier (2) indicate that acyl side chain length and unsaturation are important parameters in determining the stability of lipid bilayers.

Hemoglobin leakage from erythrocytes. Hemoglobin leakage from sheep and horse erythrocytes was determined after 10 min of incubation in isotonic buffer solutions of pH comparable to those used in the beet root experiments. Results for sheep erythrocytes are shown in Figure 5. At pH 2.0, buffered samples showed about 40% of maximum pigment leakage. Organic solvents did not greatly increase the leakage of pigment, except for petroleum hydrocarbon, which caused a maximum of 55% leakage. At pH 4.0 the differences were much more pronounced. At this pH the buffered red cells exhibited less pigment leakage than at pH 2.0, but about 20% more than was observed with betacyanin leakage from beet root cells. Methanol and ethanol did not increase pigment leakage from erythrocytes, but petroleum hydrocarbon showed nearly twice as much leakage as buffer incubated samples (40% *vs.* 20%). At pH 6.8, untreated and ethanol treated samples showed no pigment leakage, while methanol and petroleum hydrocarbon caused 8 to 10% leakage. At pH 10.0, there was virtually no leakage from samples with alcohols, but the ones incubated with petroleum hydrocarbon showed almost as much pigment loss as the C/M exposed samples (>80%).

Results with horse erythrocytes (Fig. 6) were similar to those with sheep erythrocytes with some notable exceptions. At pH 4.0, no hemoglobin leaked from buffered samples and very little leakage was observed with methanol or ethanol. Petroleum hydrocarbon, however, caused even more leakage than at pH 2.0. At pH 6.8, neither the buffered nor methanol or ethanol samples showed any pigment leakage, while petroleum hydrocarbon caused about 10% of maximum pigment leakage. At pH 10.0 there was no pigment leakage from buffered samples and very little from methanol or ethanol, but the leakage caused by petroleum hydrocarbon rose to 35%. This was less than half the damage observed in sheep erythrocytes with the same solvent.

The lipid compositions of membranes from sheep and horse erythrocytes differ considerably. The predominant phospholipids in sheep erythrocyte membranes have been reported to be SP (51.0%) and PE (26.2%) (6). We confirmed the absence of PC by isolating sheep erythrocyte ghosts, extracting the lipids with C/M, and analyzing them by thin-layer chromatography (TLC) (Jacobsohn, M.K., Lehman, M.M., and Jacobsohn, G.M., unpublished observations). Horse erythrocyte membranes contain 42.4% PC, 13.5% SP and 22.4% PE (6). The remainder of the phospholipid was mainly phosphatidylserine (PS). We measured cholesterol extractability from MLV of SP and mixtures of SP with PE or PC plus equimolar amounts of sterol and compared the data to hemoglobin leakage from erythrocytes.

Data on petroleum hydrocarbon extraction of cholesterol from vesicles of SP are shown in Figure 7. A mixture of SP/DOPE/cholesterol (1:1:2, molar ratio) allowed petroleum hydrocarbon to extract more than 480 μg or 95% of the sterol at pH <6.8, 460 μg or 92% at pH 6.8 and 250 μg or 50% at pH 10.4. The latter was unexpected because neither DOPE nor SP vesicles lost more than 10% of their cholesterol to petroleum hydrocarbon at this pH (Fig. 7). It should be pointed out, however, that there was considerable variation in the amount of sterol extracted at pH 6.8 from vesicles of SP and DOPE. Values ranging from a high of 92% to a low of 16% were observed. We

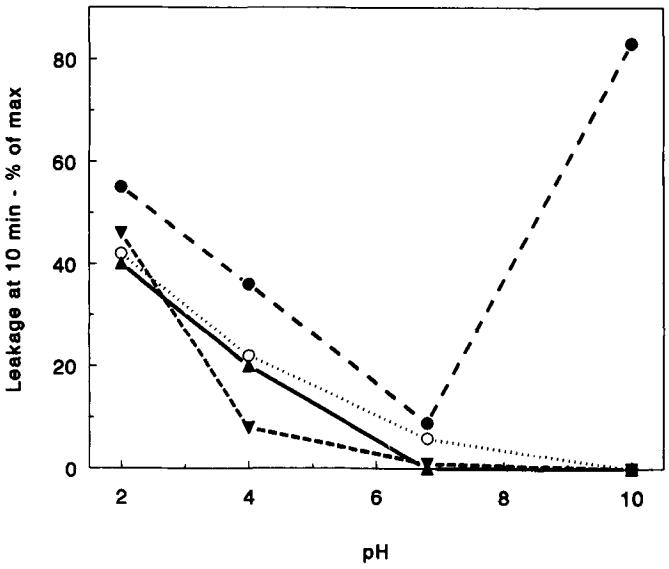

FIG. 5. Effect of solvents on hemoglobin leakage from sheep erythrocytes as function of pH at 10 min. Washed sheep erythrocytes were incubated in isotonic buffers of varying pH with or without an organic solvent. Organic solvents were present to the extent of 10% the volume of suspending media. Hemoglobin in the buffer was measured by absorbance at 590 nm and compared to similar leakage caused by chloroform/methanol, taken as 100%. Conditions are described in Materials and Methods. All points are averages for three independent assays. Standard deviations are within symbols or are shown by vertical bars. ▲, Buffer alone; ○, methanol; ▼, ethanol; ●, petroleum hydrocarbon.

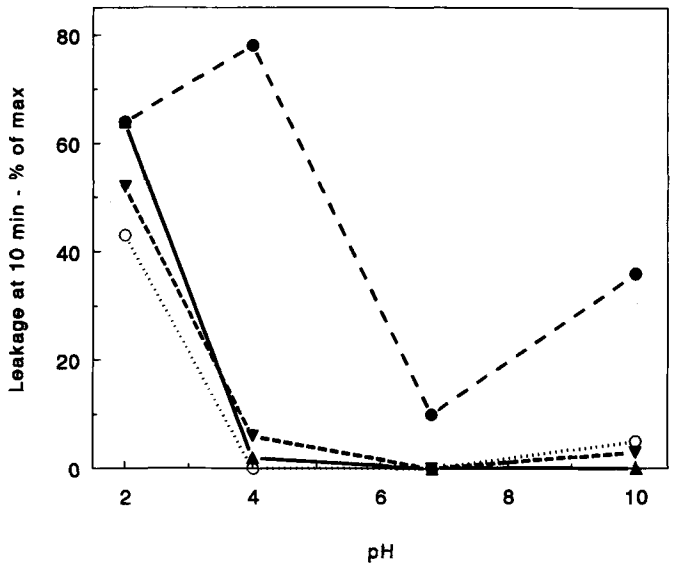

FIG. 6. Effect of solvents on hemoglobin leakage from horse erythrocytes as function of pH at 10 min. Washed horse erythrocytes were incubated in isotonic buffers of varying pH. Remainder of legend as in Figure 5. ▲, Buffer alone; ○, methanol; ▼, ethanol; ●, petroleum hydrocarbon.

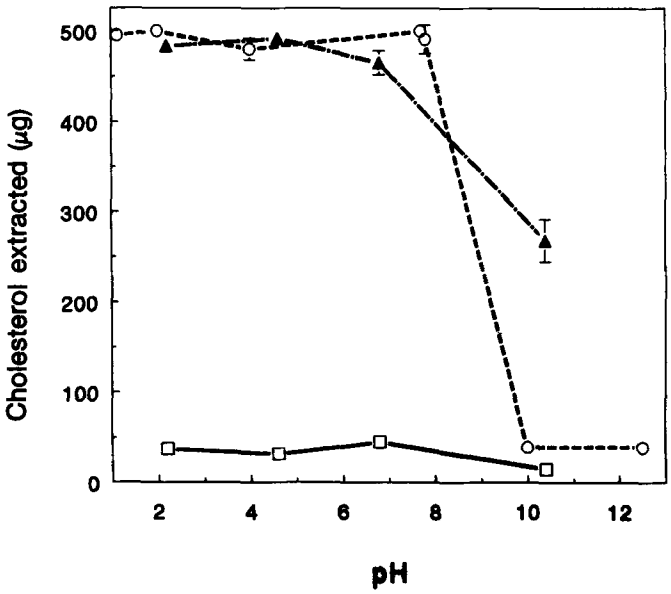

FIG. 7. Cholesterol extraction from MLV of SP/cholesterol (1:1, molar ratio), DOPE/cholesterol (1:1, molar ratio), and SP/DOPE/cholesterol (1:1:2, molar ratio) as function of pH of suspending media. Legend as in Figure 3. □, SP; ○, DOPE; ▲, SP/DOPE.

did observe consistency among the samples of any one batch of MLV. The differences suggested that the lipids did not always assume the same initial phase state (see below). MLV of SP/DOPC/DOPE/cholesterol (0.5:0.5:1:2, molar ratio) showed that 60 to 75 μg or 12 to 15% of the sterol was extracted with petroleum hydrocarbon at pH values below 10 and only 10 μg or 2% at pH 10.4 (Fig. 3).

The results of the three experiments revealed a number of interesting similarities and differences among the cell types. All of the cells displayed extensive pigment leakage at pH 2.0, and the presence of solvent tended to increase the amount of pigment lost. The beet cells were relatively insensitive to pH damage above pH 2.0, while sheep erythrocytes were stable only in the physiological range. Horse erythrocytes were more sensitive to pH changes than beet root cells, but less sensitive than sheep erythrocytes.

Extractability of cholesterol from MLV. Previous studies of cholesterol extraction from MLV made from single species of phospholipids had shown more cholesterol was extractable with petroleum hydrocarbon at low pH than at high pH (1). Phosphatidylcholines with long saturated acyl side chains were an exception in that they were unaffected by solvent extraction over the entire pH range. All of the phospholipids we examined were resistant to solvent extraction at high pH. While the pattern of pigment leakage from beet root cells with petroleum hydrocarbon followed the pattern of cholesterol loss from MLV, sheep and horse erythrocytes were much more sensitive to petroleum hydrocarbon extraction at high pH. We thought that the differences might be related to the lipid composition of the membranes.

Since lipid conformations, such as reverse hexagonal, (H_{II}) phases, would be expected to display different patterns of cholesterol extraction than bilayer phases, the amount of cholesterol removed from each of the MLV at each step of the extraction process was quantitated over the range of pH values used in the whole cell studies. Figure 8A shows the extraction pattern for cholesterol alone in various buffers. The first extraction removed 90% or more sterol from the buffers, with most of the remainder in the second extraction. The pH of the buffer had no effect upon the amount of cholesterol removed in each extraction. Figure 8B gives the extraction pattern for vesicles of SP/DOPE/cholesterol (1:1:2, molar ratio). At pH 6.8 and below, the extraction patterns for MLV that lost large amounts of sterol are very similar to those of cholesterol alone, with 68 to 77% of the sterol removed in the first extraction and indicating that sterol was accessible to the solvent at the outset. At pH 10.4, the pattern changed, with 17% of the cholesterol removed in the first extraction and 3 to 5% removed in each of the subsequent extractions. Elevation of pH rendered the lipids less accessible to the solvent. As noted earlier, at pH 6.8 there was variability in the amount of sterol extracted from SP/DOPE vesicles, but there was consistency with each subsequent extraction from the same vesicles. The results for DOPC/DOPE vesicles are shown in Figure 8C. For pH 2.2, 4.6 and 6.8, the results are similar to those observed with SP/DOPE at pH 10.4, Figure 8B. Fifteen to 22% of the sterol was removed in the first extraction, with an additional 5 to 8% being removed in each of the subsequent extractions. Relatively little sterol was exposed to the solvent initially, and the solvent itself did not make the lipid more accessible. At pH 10.4, the amount of sterol removed in each of the three extractions was virtually the same, less than 1% of the total. Figure 8D shows the results for vesicles of SP/DOPC/DOPE/cholesterol (0.5:0.5:1:2, molar ratio). At pH 2.2, 4.6 and 6.8, the amounts of cholesterol removed by each extraction were small but increased gradually with subsequent extractions, indicating that the lipid structure was altered by the solvent. At pH 10.4, the

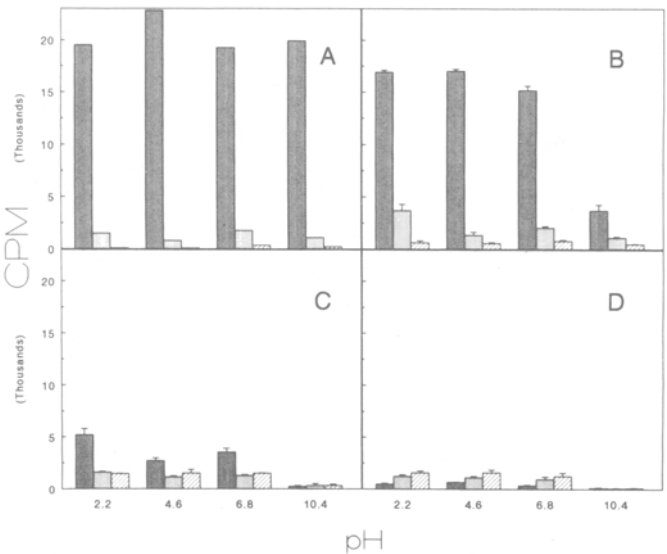

FIG. 8. Cholesterol removal from buffer and from suspensions of MLV by three successive petroleum hydrocarbon extracts as function of pH. Extractions by the organic solvent were assayed separately and are shown by vertical bars. A. Buffer only; B. MLV prepared from SP/DOPE/cholesterol (1:1:2, molar ratio); C. MLV prepared from DOPC/DOPE/cholesterol (1:1:2, molar ratio); and D. MLV prepared from SP/DOPC/DOPE/cholesterol (0.5:0.5:1:2, molar ratio). Crossed-hatched bars, 1st extraction; narrow lined bars, 2nd extraction; wide-lined bars, 3rd extraction.

amounts extracted were very low and did not increase from the first to the third extract. The extraction patterns at pH 10.4 indicated a more stable structure for vesicles of any given lipid composition, but the patterns at lower pH values suggest significant differences in the phase structures assumed by various combinations of lipids. SP particularly appears to promote configurations that make the hydrophobic portion of amphipathic lipids more accessible to a nonpolar solvent.

DISCUSSION

Studies on pigment leakage from beet root cells and erythrocytes show that pH can be a significant cause of damage to cell membranes. Natural membranes in the absence of solvent suffered more disruption at low pH than at high pH but none at physiological pH. In the presence of a highly nonpolar solvent, damage to cell membranes was influenced by pH, and the degree of disruption was related to the phospholipid composition of the membranes. The least disruption with organic solvents occurred at physiological pH. Solvents caused more damage to membranes containing SP than to those without this lipid and the disruption was proportional to the amount of SP in the membrane. Cholesterol extraction from MLV constructed with varying proportions of SP showed that petroleum hydrocarbon extracted more sterol from MLV at low pH than at high pH, with vesicles containing the highest proportions of SP showing the greatest loss of sterol. Vesicles in which the acyl side chains of phospholipids were equivalent in length and unsaturation were more stable than those in which the acyl side chains were different, as is evident by the difference in sterol retention among DPPC/DOPE, DOPC/DOPE and SP/DOPE vesicles. The pattern of cholesterol extraction from MLV suggests fundamental differences among bilayers formed by different combinations of phospholipids, some of which may be related to the presence of H_{II} phases. The notable difference between vesicles and cell membranes was the lack of maximum stability at physiological pH in the vesicles. None of the mixed lipid vesicles were resistant to petroleum hydrocarbon extraction of sterol at pH 6.8, whereas cells leaked a minimum of pigment at this pH.

The observation that pH and solvent induced instability of cell membranes and MLV was related to SP content was completely unexpected. Studies from other laboratories have shown that SP has as great or greater affinity for cholesterol than any other phospholipid (7–9), and even at low mole percent dehydroergosterol interacts preferentially with SP if the SP is the more fluid lipid in a mixture (10). Studies of cholesterol retention by SP vesicles reported here yielded the same result. We expected that membranes containing SP would be more stable than others since vesicles prepared entirely from SP lost almost no cholesterol by solvent extraction. The explanation of why membranes and vesicles of SP mixed with other phospholipids are more easily damaged by solvent stress at the ends of the pH scale most likely derives from disparity in their head groups coupled with differences in the conformation of their paraffin side chains.

Although the hydrocarbon portion of sphingosine is structurally similar to the acyl group at C-1 of the glycerol moiety of glycerophospholipids, it is shorter and carries a *trans* double bond at C-4. Further, the fatty acid attached to the amine group of sphingosine, which is analogous to the C-2 group of glycerophospholipids, can vary, but the predominating species reported from a variety of mammalian sources are 16:0, 18:0, 18:1, 20:4 and 22:6 (11). In most glycerolipids, the substituent on C-2 is an 18:1 or 18:2 fatty acid (11). The differences in hydrocarbon chain length and unsaturation may contribute to structural instability in SP-containing mixed lipid structures.

Another factor which may be involved in instability of bilayers containing SP is the asymmetric distribution of lipids within membranes. Weakly acidic phospholipids, such as PS, tend to accumulate on the basic side of a transmembrane gradient (12). In the absence of a gradient, the distribution of lipids in large vesicles is thought to be equal (13–15). Since the vesicles in the present investigation were prepared and processed in buffers of uniform pH and ion concentration and did not contain acidic phospholipids, it is unlikely that asymmetry in lipid distribution was induced. In our experiments with whole cells, pH induced lipid shifts within membranes may have occurred because of their PS content. Exposure to alkaline buffers would create pH gradients which can cause shifts in lipid distribution. Of the cells examined, beet membranes contain the highest proportion of PS, but these membranes were least affected by high pH. Thus it appears unlikely that shifts in lipid distribution within membranes would be a major cause of damage.

Indication that different phase states are assumed by the various mixed lipids in MLV was obtained from the extraction studies, based upon the following assumptions. If the phospholipids were initially in an H_{II} phase, then petroleum hydrocarbon would have ready access to sterol intercalated among the acyl side chains and to the side chains themselves. The partition coefficient for cholesterol would be close to that expected for cholesterol without phospholipid and the pattern of extraction should be similar to that of cholesterol alone in buffer. If the lipids were in a stable bilayer, each solvent extraction should remove small but constant amounts of cholesterol because the head groups of the phospholipids would protect the sterol. If the bilayer had an unstable structure, the head groups of the phospholipids might have gaps between them, or hydrogen bonding of head groups might draw a limited portion of the phospholipid into an H_{II} phase. Rand *et al.* (16) have described such behavior for mixtures of DOPC and DOPE at neutral pH. In such cases, the initial extraction with non-polar solvent would be expected to remove a relatively small amount of sterol from the vesicles, but invasion of solvent into the core of the bilayer would cause disordering, and more lipid would be removed with each subsequent extraction.

Data obtained for petroleum hydrocarbon extraction of cholesterol from mixed lipid vesicles exhibited all of the predicted patterns. Cholesterol was extractable from SP/DOPE vesicles in the same manner as from buffer, indicating that the hydrophobic portions of the vesicles were readily accessible to the solvent. At pH 10.4, the amount of extractable cholesterol was reduced, suggesting that the sterol had become inaccessible to the solvent, *i.e.*, that the orientation of the phospholipid phase had assumed a bilayer structure. Vesicles of DOPC/DOPE at pH 6.8 and below were similar to SP/DOPE vesicles at pH 10.4, except that the third extraction consistently

removed somewhat more sterol than the second extraction. This pattern is seen more clearly in the SP/DOPC/DOPE vesicles at pH 6.8 and below where the amounts increased slightly from the 1st to the 3rd extraction. At pH 10.4, only small amounts of sterol could be extracted, demonstrating that these vesicles were extremely resistant to solvent extraction. The present experiments provide, for the first time, evidence that SP promotes exposure of hydrophobic elements when it is combined with other lipids at a neutral pH or below.

The most striking observation was the stability of biological membranes at physiological pH compared to mixed lipid vesicles of defined composition. The MLV examined in these experiments lacked PS and trace phospholipids, and the fatty acyl composition of the major lipids was not equivalent to that of native membranes. While these discrepancies may account for some of the differences between MLV and cell membranes, we consider this unlikely to be the whole explanation. Evidence obtained in the present studies suggests that other factors, including proteins, may be responsible for the differences.

Pigment leakage from whole cells caused by petroleum hydrocarbon at pH 2 and at pH 10 was greater than would have been predicted from the cholesterol loss from analogous lipid vesicles. DOPC/DOPE vesicles lost less than 30% of their cholesterol at pH 2 upon extraction with petroleum hydrocarbon, but beet cells lost twice that percentage of pigment in the presence of solvent. The nearly complete extraction of pigment from erythrocytes observed at pH 2.0 was probably due to a combination of denaturation of membrane proteins and changes in lipid configuration. The observations with horse erythrocytes at pH 4 and 10 are also instructive. MLV with a lipid ratio similar to that of horse erythrocyte membranes lost about 20% of their cholesterol to the solvent at pH 4 but the erythrocytes leaked nearly 80% of their pigment under the same conditions. At pH 10, cholesterol extraction from MLV was negligible, but loss of pigment from erythrocytes was close to 40% of maximum. Loss of pigment from sheep erythrocytes was 80% of maximum at pH 10. If the effect of high pH is to denature protein but to maintain the lipid bilayer intact, petroleum hydrocarbon should not cause more pigment leakage than methanol and ethanol. Although petroleum hydrocarbon is a better solvent for the alkyl portion of membranes than alcohols, the negatively charged head groups at high pH would prevent access of petroleum hydrocarbon to the core of the membrane. Damage to membrane proteins induced by pH may influence the conformation of lipids to make the paraffin chains more accessible to a non-polar solvent.

Liu *et al.* (15,17) have noted that DOPE/oleic acid liposomes are stable over a wide range of pH and salt concentration when incubated with human plasma. They suggested that proteins from plasma insert into the core of lipid bilayers and found evidence of increased microviscosity in the core when protein was present. Hui and Sen (18) have found that lipids with non-cylindrical molecular shapes are associated with a high protein content of membranes. Sunamoto *et al.* (19) have reported that a PC analog of SP facilitated the insertion of glycoprotein into dimyristoyl phosphatidylcholine vesicles and also reduced the fluidity at the bilayer surface. Studies of fibroblasts by fluorescence recovery after photobleaching showed that lateral diffusion of phospholipids was nearly ten times slower in protein-containing membranes than in pure liposomes and that lipid diffusion in membranes is limited to the bulk phase between protein domains (20). Boggs *et al.* (21) have shown that myelin basic protein, lipophilin, preferentially binds phosphatidylserine in mixed PS-PC bilayers. Evidence suggests that proteins interact selectively with phospholipids, but once the proteins are in place, they exert an ordering effect on lipids that extends beyond their immediate domain. Changing the conformation of a protein by raising or lowering the pH from its optimal range may potentiate the formation of lipid phase structures that expose the hydrophobic portion of the bilayer to external influences.

The study of mixed lipid vesicles has proved to be a useful model in interpreting some of the characteristics of cell membranes under pH or solvent stress, but it has also underscored the limitations of extrapolating the properties of lipid bilayers to the dynamics of biological membranes. Predictions about the properties of membranes based on studies of single species of phospholipids or even combinations of two or more lipids are not always an indicator of how a cell membrane will behave. Other factors, most likely proteins, may modify lipid interactions, but the role of proteins *vis-a-vis* lipid structure is not sufficiently understood.

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Evaluation of an Oleic Acid Water-in-Oil-in-Water-Type Multiple Emulsion as Potential Drug Carrier *via* the Enteral Route

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A water-in-oil-in-water (W/O/W) emulsion composed of oleic acid was used as a carrier of carboxyfluorescein (CF) *via* the enteral route, as a model for future drug transport. The absorption of CF in the small intestine of rats given the emulsion (W/O/W group) was compared with the absorption in a group administered CF alone (CF group), and a group administered a mixed micelle of oleic acid and a surface-active agent in CF solution (MM group). Higher amounts of CF were absorbed in the W/O/W and MM groups than in the CF group. At 120 min, the amount of CF remaining in the intestinal tract was smaller in the MM group than in the W/O/W group. In the early period, CF excretion into bile was higher in the MM group than in the W/O/W group, but from 120 to 360 min, CF excretion in the W/O/W group was higher than in the MM group (non-specific). The blood CF level was significantly higher at 240 and 360 min in the W/O/W group than in the other two groups. The highest concentration in lymph was found in the W/O/W group. The W/O/W emulsion was considered superior to the micelles because it maintained a higher blood level of CF over long periods and transferred it to the lymph. This suggests that the W/O/W emulsion is applicable as a drug carrier *via* the enteral route. *Lipids 27,* 702–705 (1992).

Water-in-oil-in-water (W/O/W)-type multiple emulsions are a system of dispersed oil drops containing even smaller water droplets. Scientists working in the pharmaceutical field have been particularly interested in the development of slow-release drug delivery systems (1,2) or in selectively transferring drugs into the lymphatic system using W/O/W emulsions (3). However, there have been few applications of this type of emulsion as a drug carrier *via* the enteral route. Only Engel *et al.* (4) have attempted to enclose insulin in a W/O/W emulsion to improve intestinal absorption. In the present study, carboxyfluorescein (CF) and a W/O/W-type multiple emulsion composed of oleic acid were used as a model for drug delivery. The potential usefulness of the emulsion as a carrier was evaluated.

MATERIALS AND METHODS

Materials. CF and oleic acid were purchased from Eastman Kodak Co. (Rochester, NY) and Wako Pure Chemical Co. (Osaka, Japan), respectively. Glycerol fatty acid ester (GE; Sunsoft No. 818 NSM, Taiyo Chemical Co., Yokkaichi, Japan) and polyoxyethylene sorbitan monolaurate (Tween 20) (PSM, Wako Pure Chemical Co.) were used as surface agents. Both are currently used as food additives.

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Abbreviations: AUC, area under the concentration-time curve; CF, carboxyfluorescein; C_{MAX}, maximum concentration; GE, glycerol fatty acid ester; Kel, constant elimination rate; MM, mixed micelles; PSM, polyoxyethylene sorbitan monolaurate (Tween 20); t-MAX, time of maximum concentration; W/O/W, water-in-oil-in-water.

Preparation of emulsions. To prepare the W/O emulsion, 6 mL of CF solution containing phosphate buffer (pH 7.4) was added to a mixture of oleic acid and GE (CF solution/oleic acid/GE, 3:5:2, vol/vol/vol) and stirred for about 15 min in an ice-bath using a hand mixer (below 5°C). The W/O emulsion (7 mL) was dispersed in 93 mL of 0.2% saline solution containing 1.0% polyoxyethylene sorbitan monolaurate (PSM) and then stirred with a Bio-Mixer (higher speed mixer, Nippon Seiki Co., Tokyo, Japan, at 5°C, 6000 rpm) for about 5 min. To determine the total CF content of the W/O/W emulsion, 0.1 mL of 12.5% Triton X-100 was added to 0.1 mL of the emulsion, which was then acidified with 3 mL of 1 N HCl. CF was extracted with 6 mL isoamyl alcohol. After centrifugation, 5 mL of the organic phase was mixed and shaken with 4 mL of $Na_2CO_3/NaHCO_3$ buffer solution (pH 10). The aqueous phase was separated by centrifugation and measured fluorometrically. Then the W/O/W system was filtered through a 0.22-μm microfilter (Millex-GV, Nippon Millipore Industrial Co., Yonezawa, Japan) to separate the external water and the particles. The CF concentration in the separated external water was measured. Fluorescein in the CF solution was measured using a Shimazu Spectrofluorometer (Kyoto, Japan) at an emission wavelength of 515 nm and an excitation wavelength of 490 nm.

A mixed micelle solution of oleic acid and one of the surface-active agents was prepared as follows. Oleic acid (3.5 mL) was added to 96.5 mL of solution dissolved in phosphate buffer (pH 7.4) containing PSM (1.0%). The mixture was stirred with a Bio-Mixer for the same period of time as in the preparation of the W/O/W emulsion (at 5°C, 6000 rpm).

Absorption experiments. Male Wistar rats (weighing 180–200 g) were divided into three groups according to the mode of CF delivery. The first (CF group) consisted of rats given CF dissolved in phosphate buffer solution. The second group contained rats given CF in the W/O/W emulsion (W/O/W group), and the third CF in a solution with mixed micelles of oleic acid and surfactant (MM group).

The rats were then anesthetized intraperitoneally with pentobarbital (2.5 mg/100 g). The bile duct was cannulated with a polyethylene tube (i.d. 0.28 mm, e.d. 0.61 mm, Becton Dickinson Co., Parsippany, NJ) and bile was collected to avoid any reabsorption of CF in the bile. A similar-sized polyethylene tube for collection of blood was inserted into a femoral artery and then tunneled through the subcutaneous tissue to exit at the back. A polyethylene tube (i.d. 1.25 mm, e.d. 2.00 mm, Atom Co., Tokyo, Japan) was also inserted into the duodenum, and the distal end of the ileum was ligated. The surgical procedure was completed within 40 min, and then the rats were placed in Bollman cages. They remained anesthetized for about 50 min.

The test solutions, each containing 0.20 mg of CF in 5 mL, were introduced into the duodenum. Bile and blood samples were collected periodically.

Blood and bile assays for CF. Blood (0.04 mL) was mixed with 4 mL of isotonic phosphate-buffered saline, pH 7.4, containing 0.01% potassium chloride, and centrifuged to remove red blood cells (5). The concentration of CF in the supernatant was determined by the fluorometric procedure described above. Bile was diluted with phosphate buffer (pH 7.4) and assayed in the same manner.

Assay of CF remaining in the small intestine. Rats were killed periodically and the small intestine was excised from each animal. The intestine was then homogenized and the remaining CF was extracted and assayed by the method of Hashida *et al.* (6).

CF concentration in lymph. The thoracic duct was cannulated, and CF was given in the same manner as described above. Lymph from the thoracic duct was obtained for assay as described above, except that 0.1 mL of lymph was collected.

Effect of changes in oleic acid concentration on absorption of CF. Alteration of the volume of the W/O emulsion varied the concentration of oleic acid (40, 80, 120 mM) and thus modified the concentration of entrapped CF. The total CF concentration in the W/O/W emulsion was maintained at 0.02 mg/mL, and the volume administered was 5 mL.

RESULTS

The diameter of the W/O/W emulsion droplets was measured by microphotography, it ranged from 0.25 to 20 μm (Fig. 1, Table 1). Each droplet contained many smaller water droplets. The entrapping efficiency of CF was 89.5 ± 4.0% (mean ± SD) and did not change when the emulsion was stored at 5°C for 7 d.

Concentration of CF in blood. The CF concentrations in the blood of rats in the three groups were compared. The CF level increased more steeply, and blood levels were significantly higher in the MM and W/O/W groups than in the CF group. In the W/O/W group, the blood levels of CF at 240 and 360 min were higher than in the other two groups (Fig. 2).

TABLE 1

Size of the W/O/W Emulsion Particles[a]

Size (μm)	Frequency (%)
0.5	11.4
0.5–1.0	21.1
1.0–2.0	37.8
2.0–3.0	20.2
3.0–4.0	5.6
4.0–5.0	1.6
5.0–6.0	1.8
6.0	0.5

[a]The sizes of 1200 emulsion particles were measured by microphotography.

The maximum concentration (C_{MAX}) and area under the concentration-time curve (AUC) were significantly higher in the W/O/W and MM groups than in the CF group, but there was no significant difference between the former two groups (Table 2).

Amount of CF secreted into bile. No difference in bile volume was found among the three groups (Table 3). The W/O/W and MM groups delivered CF to bile more efficiently than the CF group. In the early period (0–120 min), the amount of CF in the MM group was higher than that in the W/O/W group. From 120 to 360 min, the W/O/W group had higher levels of CF than the MM group, but not to a significant extent (Fig. 3, Table 4).

Amount of CF remaining in the small intestine. The amount of CF remaining in the small intestine was lower in the W/O/W and MM groups than in the CF group

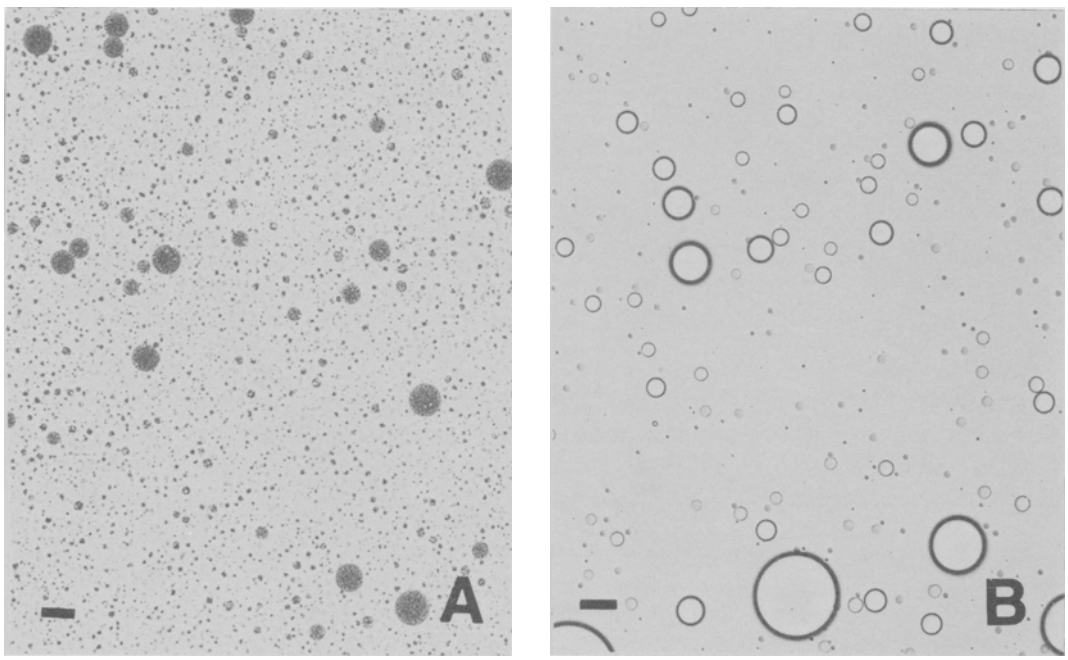

FIG. 1. Micrographs: A. Oleic acid water-in-oil-in-water emulsion. B. Mixed micelle of oleic acid. The bar is 20 μm.

TABLE 2

Maximum Blood Carboxyfluorescein (CF) Concentration (C_{MAX}) and Area Under the Concentration Time Curve (AUC)[a]

	Group		
	CF	W/O/W	MM
C_{MAX} (ng/mL)	27.8 ± 3.7[bc]	196.5 ± 27.6	260.2 ± 38.8
AUC 0-360 min (ng•min/mL)	7107.2 ± 884.0[bc]	32212.0 ± 4475.8	24657.7 ± 3307.8

[a]Maximum blood CF concentration and area under the concentration time curve for 6 h after administration. Values represent means ± SEM of five animals.
[b]The values are significantly different from the W/O/W group ($P < 0.01$).
[c]The values are significantly different from the MM group ($P < 0.01$).

TABLE 3

Bile Volume Collected (mL)

	Time (min)				
Group	0-30	30-60	60-120	120-240	240-360
CF[a]	0.40 ± 0.06	0.42 ± 0.04	0.67 ± 0.07	1.30 ± 0.20	1.10 ± 0.23
W/O/W[a]	0.39 ± 0.01	0.35 ± 0.05	0.55 ± 0.03	0.85 ± 0.13	0.65 ± 0.06
MM[a]	0.41 ± 0.01	0.40 ± 0.06	0.68 ± 0.06	1.10 ± 0.06	0.78 ± 0.04

[a]CF, carboxyfluorescein; W/O/W, water-in-oil-in-water; MM, mixed micelles.

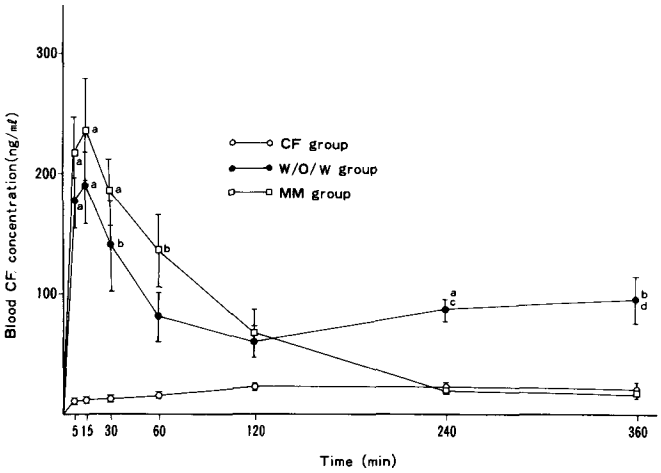

FIG. 2. Carboxyfluorescein (CF) concentration in blood. Rats were divided into three groups according to the mode of CF delivery. (○), CF dissolved in buffer solution (CF group); (●) CF in water-in-oil-in-water (W/O/W) emulsion (W/O/W group); (□) CF in a solution with mixed micelles of oleic acid and surfactant (MM group). Values represent means ± SEM of five animals. Statistical analysis was done by Student's *t*-test. Significantly different from CF group, $^aP < 0.01$, $^bP < 0.05$; from MM group, $^cP < 0.01$, $^dP < 0.05$.

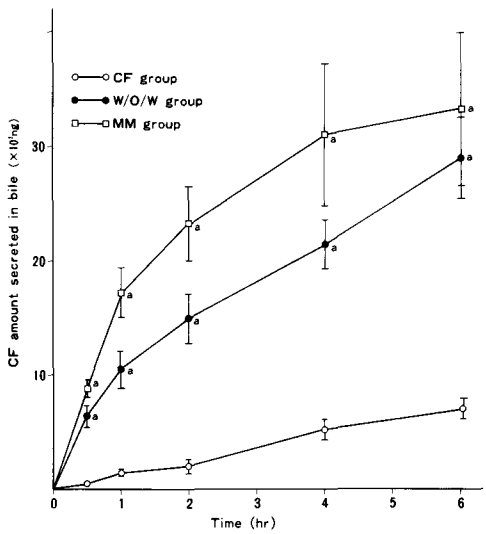

FIG. 3. Amount of carboxyfluorescein (CF) secreted into bile. (○), CF group; (●), water-in-oil-in-water group; (□), mixed micelle group. Values represent means ± SEM of five animals. Significantly different from CF group $^aP < 0.01$, $^bP < 0.05$.

throughout the course of the experiment. At 2 h after administration, the MM group had a lower amount of CF than the W/O/W group ($P < 0.05$), but no significant differences were observed at subsequent time points (Fig. 4).

Amount of CF in lymph. The CF content of lymph was significantly higher in the W/O/W group than in the other two groups, except at 4-6 h (Table 5, Fig. 5). However, throughout the experiment, the CF concentration in lymph was lower than that in plasma in all groups.

Effect of oleic acid concentration on absorption. In the early period, the CF level was elevated in each group (40,

TABLE 4

Amount of CF[a] Secreted into Bile (× 10^3 ng)[b]

	Group		
Time	CF	W/O/W[a]	MM[a]
0-120	2.11 ± 0.34[c]	14.96 ± 2.16	22.72 ± 3.20
120-240	2.58 ± 0.43[d]	6.59 ± 0.87	4.72 ± 0.84
240-360	2.45 ± 0.56	8.25 ± 3.02	2.26 ± 0.57

[a]CF, carboxyfluorescein; W/O/W, water-in-oil-in-water; MM, mixed micelles.
[b]Values represent means ± SEM of five animals.
[c]The value is significantly different from the MM and the W/O/W group ($P < 0.01$).
[d]The value is significantly different from the W/O/W group ($P < 0.05$).

TABLE 5

Relationship Between Oleic Acid Concentration and Absorption Efficiency[a]

Oleic acid concentration	C_{MAX} (ng/mL)	AUC_{0-360} (ng·min/mL)	t-MAX (min)	K_{el} (h^{-1})
40 mM	121.7 ± 25.3	11399.8 ± 3266.3	21.0 ± 13.1	0.659 ± 0.068[b,d]
80 mM	144.4 ± 42.0	17102.6 ± 4075.6	16.3 ± 5.2	0.391 ± 0.029[c]
120 mM	125.0 ± 9.0	22412.9 ± 2542.8	17.5 ± 4.3	0.258 ± 0.032

[a]Values represent mean ± SEM of four animals. The values are significantly different from the 120 mM group ([b]$P < 0.01$, [c]$P < 0.05$). [d]The values are significantly different from the 80 mM group ($P < 0.05$). Abbreviations: C_{MAX}, maximum concentration; AUC, area under the concentration-time curve; t-MAX, time of maximum concentration; Kel, constant elimination rate.

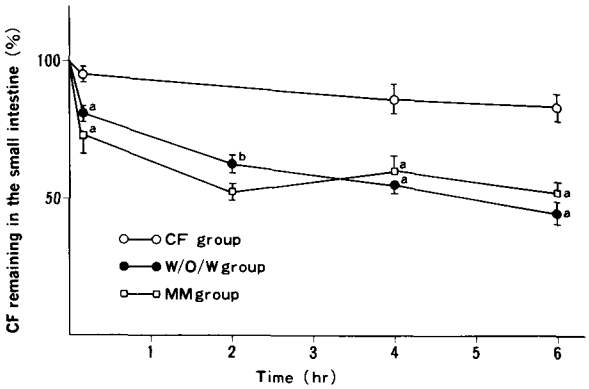

FIG. 4. Amount of carboxyfluorescein (CF) remaining in the small intestine. (○), CF group (n = 5); (●), water-in-oil-in-water group (n = 5–6); (□), mixed micelle group (n = 5–7). Significantly different from CF group [a]$P < 0.01$, from MM group [b]$P < 0.05$.

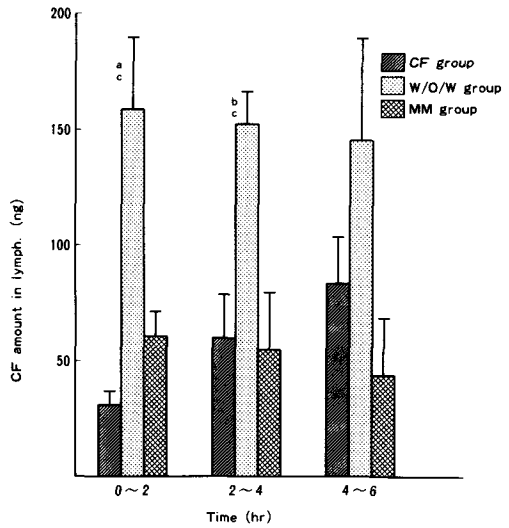

FIG. 5. Amount of carboxyfluorescein (CF) in lymph. Values represent means ± SEM of four animals. Significantly different from CF group [a]$P < 0.01$, [b]$P < 0.05$; from MM group [c]$P < 0.05$.

80, 120 mM) with no significant differences among the groups. After 120 min, in the 80 mM and 120 mM oleic acid groups, the levels of CF in blood were significantly higher than in the CF group, but the 40 mM group showed no difference from the CF group after 2 h (Fig. 6). The C_{MAX} and t-MAX values for the three groups did not differ, but the AUC increased in proportion to the concentration of oleic acid. Kel (constant elimination rate) fell significantly in proportion to the concentration of oleic acid (Table 6).

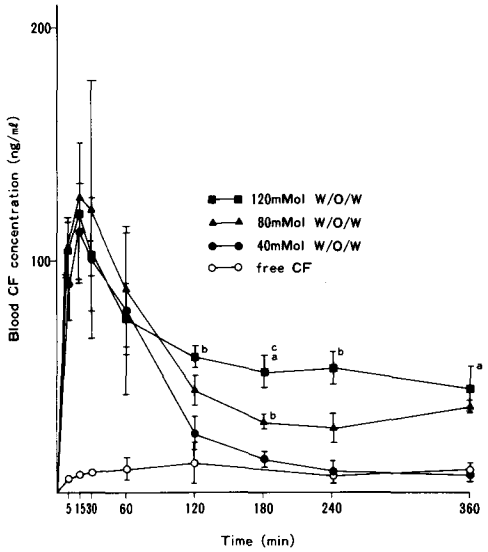

FIG. 6. The effect of changes in the oleic acid concentration on the absorption of carboxyfluorescein (CF). The concentration of oleic acid as a constituent of the water-in-oil-in-water emulsion was varied: (■), 120 mM; (▲), 80 mM; (●), 40 mM; (○), free CF. Total quantity of CF was controlled at 0.1 mg/5 mL/rat. Significantly different from 40 mM [a]$P < 0.01$, [b]$P < 0.05$; from 80 mM [c]$P < 0.05$.

TABLE 6

Lymph Volume Collected (mL)

Group	Time (h) 0–2	2–4	4–6
CF[a]	0.90 ± 0.01	0.61 ± 0.60	0.43 ± 0.12
W/O/W[a]	0.57 ± 0.07	0.57 ± 0.08	0.48 ± 0.13
MM[a]	0.43 ± 0.12	0.64 ± 0.04	0.48 ± 0.06

[a]CF, carboxyfluorescein; W/O/W, water-in-oil-in-water; MM, mixed micelles.

DISCUSSION

A W/O/W-type multiple emulsion was used as a carrier of CF *via* the enteral route for the following reasons. Multiple emulsions already have been used orally (7). Because of their low viscosity, they are easy to handle and to drink (8). Furthermore, drugs can be enclosed both easily and efficiently with multiple emulsions (9). The behavior of an emulsion is largely dependent on the lipid that constitutes the oil phase (8). If the emulsion is composed of unsaturated fatty acids, an absorption-promoting action may be obtainable (9–11). W/O/W emulsions enter the lymphatic system easily and reach regional lymph nodes selectively (11).

In the present study, when an oleic acid W/O/W emulsion was administered into rat small intestine, a larger amount of CF was absorbed in the W/O/W group than in the CF group. The MM group had more CF left in the intestinal tract at 120 min, but no significant differences were observed at later time points. The W/O/W group was superior to the MM group in transferring CF to the blood at the later time points, and transferred CF to the lymphatic system more efficiently than the other two groups.

The details of the absorption promoting action remain obscure. The number and size of particles may affect the absorption of CF. More particles were found in W/O/W emulsion and the particle diameter was smaller; therefore, the W/O/W emulsion may be more advantageous than micelles. Blood levels of CF dropped quickly when an oleic acid concentration of 40 mM was used. This decline may be related to the number of particles.

Some anticancer drugs produce considerable side-effects following intravenous administration, and are difficult to absorb *via* the digestive tract. If such a drug could be enclosed in oleic acid W/O/W emulsion and delivered to the liver and the lymphatic system efficiently, then treatment and prophylaxis of liver metastasis and nodal involvement could be improved. Thus, for administration of such a drug, W/O/W emulsions could be more advantageous than micelles. Also, oleic acid W/O/W emulsions would be superior to micelles for lymphatic transfer of anticancer drugs.

In administering W/O/W emulsions to the digestive tract, alteration in stability due to pH, intestinal peristalsis, saliva and gastric juice pose a problem. Fusion has been shown to take place in a dopamine W/O/W emulsion (soybean oil and egg yolk lecithin) in an acid environment (13). However, W/O/W emulsions can be stabilized by gelatinization of the internal aqueous phase in the emulsion (14). Therefore, some emulsions which are stable in the stomach environment are available (13), and these could overcome the problem of instability. If such stability could be achieved for oleic acid W/O/W emulsion, it could be an excellent drug carrier.

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Omega-3 Fatty Acid and Cholesterol Content of Newly Hatched Chicks from α-Linolenic Acid Enriched Eggs

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Egg yolk was enriched with α-linolenic acid (18:3n-3) by feeding laying hens diets containing flax, canola or soybean seeds. Fertilized eggs were incubated and the fatty acid composition of whole body, liver, plasma, brain and the cholesterol content of plasma and liver tissue of the hatched chicks were studied. Eggs enriched with 18:2n-6 fatty acids by feeding hens diets containing sunflower seeds were used as the controls. Feeding flax enriched ($P < 0.05$) egg yolk and the developing progeny with 18:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3. Feeding sunflower seeds resulted in an increase ($P < 0.05$) of 18:2n-6, 20:4n-6, 22:4n-6 and 22:5n-6. The predominant polyunsaturated fatty acid of the brain was docosahexaenoic acid (22:6n-3) which was higher ($P < 0.05$) in the flax and canola fed group. The cholesterol content of the liver tissue was lower ($P < 0.05$) in chicks hatched from hens fed flax seeds. This study indicates that 18:3n-3 and 18:2n-6 in the maternal diet are potent modulators of long-chain polyunsaturated n-3 or n-6 fatty acid and of cholesterol content in the developing progeny.
Lipids 27, 706–710 (1992).

The essentiality of dietary n-3 fatty acids in the development of brain tissue is well documented (1,2). Docosahexaenoic acid (DHA) is the major n-3 polyunsaturated fatty acid (PUFA) in the brain (3). Diets deficient in n-3 fatty acids have resulted in inferior learning ability in rats (4) and neurological disorders in humans (5). The precursor of DHA is linolenic acid (LNA, 18:3n-3). LNA is desaturated and elongated to form long-chain PUFA of the n-3 series such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (6). Plant oil seeds such as flax and canola are a rich source of LNA.

The enrichment of chicken egg with n-3 fatty acids by incorporating flax and canola seeds or their oils in laying hens' diets has been well documented (7–10). During incubation of a fertile egg, the lipids of the yolk serve as an energy source and supply fatty acids for the synthesis of membrane lipids of the developing chicken embryos (11). Unlike the mammalian fetus, the chicken embryo is not dependent on maternal fatty acids through circulation. Maternal fatty acids can cross the placenta and may, for example, contribute up to 50% of fetal fatty acids during late gestation in the rat (12). Instead, we chose the hen, the fertilized egg and the hatched chick because when a fertilized egg is incubated, the developing embryo is in a "contained" environment. The laying hen, the fertilized egg and the hatched chick were considered useful to study n-3 fatty acid transfer from the egg to the developing embryo and its impact on the lipid metabolism of the progeny.

The hypocholesterolemic effect of n-3 fatty acids from fish oil (13,14) and from linseed oil has been established in human trials (15) and also in experimental animals (16). However, n-3 fatty acids in eggs and its effect on the plasma and/or tissue cholesterol content in the developing progeny has not been reported. The objective of the present study was to determine to which extent dietary n-3 or n-6 fatty acids can be incorporated into egg yolk and to examine its effect on the fatty acid and cholesterol content of the progeny (hatched chick).

MATERIALS AND METHODS

Fatty acid modulation. Single comb white leghorn (SCWL) laying hens were housed in cages (30 × 40 cm) and were assigned to test diets containing 10% ground flax seed, 10% ground canola seed or 16% ground soybean seed, and were compared to birds fed 12% sunflower seed serving as controls (Table 1). The fatty acid composition of the diets is shown in Table 2. The hens were artificially inseminated on consecutive days with 0.05 mL of pooled semen sample. Fertilized eggs were collected for six days

TABLE 1

Composition of Laying Hen Diets

Ingredients	Experimental diets			
	Flax (%)	Canola (%)	Soybean (%)	Sunflower (%)
Wheat	64.66	65.04	68.00	62.21
Soybean meal	10.28	11.05	13.16	12.01
Animal tallow	3.25	2.18	2.88	2.00
Flax seeds	10.00	—	—	—
Canola seeds	—	10.00	—	—
Sunflower seed	—	—	—	12.00
Soybean seed	—	—	16.00	—
DiCal–phos	1.00	0.98	1.00	0.98
Limestone	8.33	8.29	8.32	8.34
Iodized salt	0.29	0.28	0.28	0.29
D/L-Methionine	0.07	0.07	0.10	0.09
Layer premix[a]	2.10	2.00	2.00	2.10
Calculated composition				
Crude protein (%)	16.50	16.80	16.80	16.50
ME (kcal/kg)	2700.00	2900.00	2900.00	2747.00

[a]Layer premix supplied per kg of the diet the following: vitamin A, 8,000 IU; vitamin D_3, 1,200 ICU; vitamin E, 5 IU; riboflavin, 4 mg; calcium pantothenate, 6 mg; niacin, 15 mg; vitamin B_{12}, 10 μg; choline chloride, 100 mg; biotin, 100 μg; DL-methionine, 500 mg; manganese sulfate, 146 mg: zinc oxide, 58 mg.

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Abbreviations: ANOVA, analysis of variance; CE, eggs from hens fed diets containing ground canola seed; CAC, chicks hatched from hens fed diets containing ground canola seed; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FE, eggs from hens fed diets containing ground flax seed; FXC, chicks hatched from hens fed diets containing ground flax seed; GC, gas chromatograph; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; LNA, α-linolenic acid; MUFA, monounsaturated fatty acid(s); PUFA, polyunsaturated fatty acid(s); SBC, chicks hatched from hens fed diets containing ground soybean seed; SBE, eggs from hens fed diets containing ground soybean seed; SFC, ground sunflower; SCWL, single comb white leghorn; SFE, eggs from hens fed diets containing ground sunflower seed.

after two weeks on experimental diets and incubated in a forced-air incubator (Robbins Incubator Company, Denver, CO). The eggs were incubated at 37°C at a relative humidity of 64% and turned once per hour for eighteen days. They were then transferred to hatchers and incubated for three days at 37.2°C and at a relative humidity of 64%.

Sample collection and analyses. Eggs from laying hens fed ground flax seed (FE), ground canola seed (CE), ground soybean seed (SBE) and the ground sunflower seed (SFE) were collected and the yolks were separated, weighed and kept frozen till analysis. On the day of hatching, 10 newly hatched chicks from laying hens fed diets containing ground flax (FXC), ground canola (CAC), ground soybean seed (SBC) or ground sunflower (SFC) were weighed and killed by decapitation. Five chicks from each treatment were ground in chloroform/methanol (2:1, vol/vol) (17) solution with a polytron (Brinkman Instruments, Westbury, NY). From the remaining five chicks, blood, brain and liver tissues were collected. Blood samples were centrifuged at 3000 rpm in a Beckman centrifuge (Beckman Instruments, Mississauga, Ontario, Canada) and plasma was separated. Total lipids were extracted from yolk, whole chicks, liver, plasma and brain by the method of Folch *et al.* (17). Aliquots of the lipid extracts were dried under nitrogen and were converted to fatty acid methyl esters using a mixture of boron trifluoride, hexane and methanol (35:20:45, vol/vol/vol) (18). Fatty acid methyl esters were separated and quantified by automated gas chromatography (Model 3400, Varian Associates, Inc., Sunnyvale, CA) using a DB-23 fused capillary column (30 m × 0.25 mm). The initial column temperature was set at 70°C for 3 min, increased to 180°C at 30°C/min, and held for 10 min. Then the column temperature was elevated to 230°C at a temperature of 5°C/min and held at the final temperature for 3 min. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The fatty acid results were expressed on a percentage basis. Plasma cholesterol was measured by the enzymatic method using a commercial kit (Sigma Chemicals Co., St. Louis, MO). For liver cholesterol determination, an aliquot of the total lipid extract was dried under nitrogen along with the internal standard 5α-cholestane, and the cholesterol content was measured by gas chromatography (GC) (19).

Statistics. Data were analyzed by one-way analysis of variance (ANOVA). Significant differences between treatment means were determined with Duncan's multiple range test (20).

RESULTS

The total lipid and fatty acid composition of the diets are shown in Table 2. The only sources of n-3 or n-6 fatty acids in the diet were 18:3n-3 and 18:2n-6. The total n-3 and n-6 fatty acids in the diet were 27.2, 11.4, 5.0, 3.3% and 19.8, 25.4, 37.3, and 45.0 for flax, canola, soybean and the sunflower based diets, respectively. The fatty acid composition of egg yolk was significantly modified by the laying hen test diets (Table 3). Feeding ground flax seed enriched the egg yolk with LNA and its metabolites such as EPA and DHA resulting in the total incorporation of 8.29% of n-3 fatty acids compared with 2.83% for canola seed, 1.66% for soybean seed and 1.11% for ground sunflower seed. LNA was the major n-3 fatty acid in the yolk of hens fed diets containing ground flax or canola

TABLE 2

Total Lipid and Fatty Acid Composition of the Experimental Diets (%)

Fatty acid	Flax	Canola	Soybean	Sunflower
18:2n-6	19.82	25.46	37.30	45.01
18:3n-3	27.03	10.98	5.46	3.31
Σ Saturated	18.80	14.55	21.51	19.06
Σ Monounsaturated	33.66	48.22	35.05	32.35
Σ n-6	19.82	25.46	37.30	45.01
Σ n-3	27.26	11.43	5.00	3.31
n-6/n-3	0.73	2.23	7.46	13.59
Σ Lipids (%)	5.20	5.85	4.06	4.25

TABLE 3

Fatty Acid Composition of the Egg Yolk Lipids[a]

Fatty acid	% of total fatty acids[b]			
	FE	CE	SBE	SFE
18:2n-6	10.62 ± 0.18^{b}	11.15 ± 0.12^{b}	11.47 ± 0.16^{b}	21.77 ± 0.22^{a}
20:4n-6	0.82 ± 0.18^{c}	1.37 ± 0.08^{b}	1.98 ± 0.38^{b}	2.18 ± 0.12^{a}
18:3n-3	5.77 ± 0.09^{a}	1.37 ± 0.10^{b}	0.63 ± 0.07^{c}	0.47 ± 0.09^{c}
20:5n-3	0.18 ± 0.05^{a}	0.08 ± 0.02^{a}	nd	nd
22:5n-3	0.49 ± 0.02^{a}	0.16 ± 0.01^{a}	nd	nd
22:6n-3	1.85 ± 0.41^{a}	1.22 ± 0.03^{a,b}	1.03 ± 0.26^{b}	0.64 ± 0.26^{c}
Σ n-6	11.44 ± 0.50^{b}	12.52 ± 0.14^{b}	13.45 ± 0.41^{b}	23.95 ± 0.25^{a}
Σ n-3	8.29 ± 0.61^{a}	2.83 ± 0.17^{b}	1.66 ± 0.35^{c}	1.11 ± 0.34^{c}
n-6/n-3	1.38 ± 0.49^{d}	4.42 ± 0.19^{c}	8.10 ± 0.26^{b}	21.58 ± 0.39^{a}
Lipids	5.85 ± 0.32	5.41 ± 0.28	5.63 ± 0.23	5.88 ± 0.19

[a]Superscripts a–d: means with no common superscripts within the same rows are significantly different ($P < 0.05$). Data are presented as mean ± SEM, n = 5. FE, CE, SBE, SFE represent eggs from hens fed diets containing ground flax, canola, soybean or sunflower seeds, respectively. nd, Not detectable. Percent of total lipids is represented on the last line entry (Lipids).

[b]Laying hens fed ground flax seed (FE), canola seed (CE), soybean seed (SBE) and sunflower seed (SFE).

seed, although considerable amounts of EPA and DHA were found in these yolks. Conversely, incorporation of 12% sunflower seeds in the laying hen's diet resulted in a significant increase in 18:2n-6 and arachidonic acid (20:4n-6) at the expense of oleic acid (18:1n-9). Consequently, this resulted in a decrease in monounsaturated fatty acid (MUFA) (data not shown) content and a higher total n-6/n-3 ratio in the SFE eggs. The 20:4n-6 content of FE eggs was significantly ($P < 0.05$) lower than the canola group at 0.82 and 1.37%, respectively.

The total lipid fatty acid composition of whole body, plasma and liver tissue was modified by the egg yolk lipid composition (Table 4). Significantly higher contents of LNA and its metabolites such as EPA, DPA and DHA were found in the total body lipids, plasma lipids and liver lipids of the hatched chicks from n-3 fatty acid enriched FE, CE and SBE than those hatched from n-6 fatty acid enriched SFE eggs. The chicks hatched from FE, CE and SBE had significantly reduced 18:2n-6 and 20:4n-6 contents. In contrast, the chicks hatched from n-6 fatty acid enriched eggs (SFE) had significantly higher levels of 18:2n-6 and its metabolites, such as 20:4n-6, 22:4n-6 and 22:5n-6, in all tissues examined. The total lipid content of the whole body and the liver was not affected. However, plasma total lipids were significantly reduced in the FXC and CAC chicks when compared with SBC and SFC chicks.

The brain fatty acid composition of the newly hatched chicks is shown in Table 5. Compared to SBC and SFC chicks, CAC and FXC chicks had elevated concentration of EPA, DPA and DHA. The effect was most pronounced with respect to DHA which is the major polyunsaturated fatty acid in chick brain. The concentrations of 18:2n-6, 22:4n-6 and 22:5n-6 were significantly higher in the SFC

TABLE 4

Total Lipids and Fatty Acid Composition of Tissues of Newly Hatched Chicks[a]

Tissue	Fatty acid	% of total fatty acids			
		FXC	CAC	SBC	SFC
Whole body	18:2n-6	12.01 ± 0.18^{c}	12.96 ± 0.12^{c}	15.71 ± 0.16^{b}	22.17 ± 0.12^{c}
	20:4n-6	1.10 ± 0.21^{c}	1.22 ± 0.08^{c}	1.87 ± 0.02^{b}	2.22 ± 0.12^{c}
	22:4n-6	nd	0.14 ± 0.02^{b}	0.10 ± 0.01^{b}	0.45 ± 0.02^{a}
	22:5n-6	nd	nd	nd	0.40 ± 0.11
	18:3n-3	4.79 ± 0.20^{a}	1.55 ± 0.02^{b}	0.46 ± 0.01^{c}	0.38 ± 0.03^{c}
	20:5n-3	0.11 ± 0.05	0.13 ± 0.02	nd	nd
	22:5n-3	1.47 ± 0.02^{a}	0.19 ± 0.01^{b}	nd	nd
	22:6n-3	1.05 ± 0.41^{a}	0.84 ± 0.03^{b}	0.94 ± 0.26^{a,b}	0.92 ± 0.26^{a,b}
	Σ n-6	13.11 ± 0.50^{c}	14.32 ± 0.14^{c}	17.68 ± 0.41^{b}	25.24 ± 0.25^{a}
	Σ n-3	7.87 ± 0.50^{a}	2.71 ± 0.17^{b}	1.40 ± 0.35^{c}	1.30 ± 0.19^{c}
	n-6/n-3	1.67 ± 0.09^{d}	5.28 ± 0.12^{c}	12.62 ± 0.21^{b}	19.42 ± 0.29^{a}
	Lipids (%)	5.66 ± 0.18	4.43 ± 0.28	4.07 ± 0.23	4.43 ± 0.28
Liver	18:2n-6	10.96 ± 0.32^{c}	11.33 ± 0.12^{c}	12.71 ± 0.22^{b}	14.45 ± 0.09^{a}
	20:4n-6	4.07 ± 0.12^{c}	6.25 ± 0.08^{b}	7.10 ± 0.38^{b}	9.35 ± 0.12^{a}
	22:4n-6	nd	nd	0.22 ± 0.02^{b}	0.55 ± 0.02^{a}
	22:5n-6	nd	nd	0.32 ± 0.01^{b}	1.04 ± 0.04^{a}
	18:3n-3	2.01 ± 0.09^{a}	0.73 ± 0.10^{b}	0.26 ± 0.07^{c}	nd
	20:5n-3	0.18 ± 0.05^{b}	0.30 ± 0.05^{a}	0.31 ± 0.02^{a}	nd
	22:5n-3	0.45 ± 0.02	0.47 ± 0.01	nd	nd
	22:6n-3	5.30 ± 0.41^{a}	4.92 ± 0.03^{a}	3.87 ± 0.26^{a,b}	2.43 ± 0.26^{b}
	Σ n-6	15.03 ± 0.23^{d}	17.58 ± 0.56^{c}	20.35 ± 0.89^{b}	25.39 ± 0.58^{a}
	Σ n-3	7.94 ± 0.36^{a}	6.42 ± 0.11^{a}	4.44 ± 0.27^{b}	2.43 ± 0.15^{c}
	n-6/n-3	1.89 ± 0.24^{d}	2.74 ± 0.17^{c}	4.58 ± 0.23^{b}	10.45 ± 0.20^{a}
	Lipids (%)	13.69 ± 0.34	12.91 ± 0.46	12.14 ± 0.28	12.81 ± 0.31
Plasma	18:2n-6	20.46 ± 0.22^{c}	21.08 ± 0.27^{c}	24.26 ± 0.19^{b}	27.81 ± 0.37^{a}
	20:4n-6	3.96 ± 0.03^{d}	5.96 ± 0.10^{c}	8.00 ± 0.07^{b}	10.41 ± 0.10^{a}
	22:4n-6	nd	0.25 ± 0.01	0.26 ± 0.03^{b}	0.47 ± 0.03^{a}
	22:5n-6	nd	nd	0.26 ± 0.01	0.29 ± 0.02
	18:3n-3	4.15 ± 0.16^{a}	1.12 ± 0.03^{b}	0.72 ± 0.01^{c}	0.30 ± 0.09^{d}
	20:5n-3	0.97 ± 0.05^{a}	0.61 ± 0.02^{b}	0.43 ± 0.02^{c}	nd
	22:5n-3	2.62 ± 0.02^{a}	0.39 ± 0.01^{b}	0.35 ± 0.02^{b}	nd
	22:6n-3	3.80 ± 0.09^{a}	2.43 ± 0.16^{b}	2.23 ± 0.06^{c}	1.55 ± 0.03^{b,c}
	Σ n-6	24.42 ± 0.25^{d}	27.29 ± 0.45^{c}	32.78 ± 0.61^{b}	38.98 ± 1.45^{a}
	Σ n-3	11.54 ± 0.23^{a}	4.55 ± 0.29^{b}	3.73 ± 0.32^{c}	1.85 ± 0.32^{d}
	n-6/n-3	2.12 ± 0.14^{d}	5.99 ± 0.23^{c}	8.79 ± 0.19^{b}	21.07 ± 0.29^{a}
	Lipids (%)	0.93 ± 0.02^{b}	0.88 ± 0.49^{b}	1.27 ± 0.07^{a}	1.18 ± 0.03^{a}

[a]Superscripts a–d: means with no common superscripts within the same rows are significantly different ($P < 0.05$). Data are presented as mean ± SEM, n = 5. g/dL is represented in the last line entry (Lipids %). FXC, CAC, SBC and SFC represent chicks hatched from hens fed flax, canola, soybean or sunflower seeds, respectively. nd, Not detectable.

TABLE 5

Total Lipids and Fatty Acid Composition of Brain Tissue of Newly Hatched Chicks (%)[a]

Fatty acid	% of total fatty acids			
	FXC	CAC	SBC	SFC
18:2n-6	2.34 ± 0.04[b]	2.62 ± 0.09[b]	2.25 ± 0.25[b]	3.47 ± 0.13[a]
20:4n-6	5.77 ± 0.04[c]	7.55 ± 0.45[b]	8.74 ± 0.26[a]	9.38 ± 0.26[a]
22:4n-6	1.00 ± 0.03[c]	1.2 ± 0.03[c]	1.70 ± 0.07[b]	2.40 ± 0.12[a]
22:5n-6	1.11 ± 0.11[b]	1.13 ± 0.11[b]	1.18 ± 0.12[b]	3.77 ± 0.33
18:3n-3	0.26 ± 0.04	0.30 ± 0.04	nd	nd
20:5n-3	0.69 ± 0.05[a]	0.72 ± 0.05[a]	0.31 ± 0.11[b]	0.12 ± 0.03[c]
22:5n-3	2.04 ± 0.06[a]	2.19 ± 0.08[a]	0.31 ± 0.02[b]	nd
22:6n-3	18.67 ± 0.09[a]	17.39 ± 0.45[a]	13.21 ± 0.46[b]	10.12 ± 0.45[c]
Σ n-6	10.22 ± 0.06[c]	12.50 ± 0.56[b]	13.87 ± 0.66[b]	19.02 ± 1.01[a]
Σ n-3	21.66 ± 0.73[a]	20.60 ± 0.73[a]	13.83 ± 0.76[b]	10.24 ± 0.73[c]
n-6/n-3	0.47 ± 0.12[c]	0.61 ± 0.02[c]	1.00 ± 0.24[b]	1.86 ± 0.09[a]
Lipids	4.41 ± 0.18	3.98 ± 0.08	4.36 ± 0.06	4.21 ± 0.16

[a]Superscripts a–c: means with no common superscripts within the same rows are significantly different ($P < 0.05$). Data are presented as mean ± SEM, n = 5. FXC, CAC, SBC and SFC represent chicks hatched from hens diets containing ground flax, canola, soybean or sunflower seeds. Percent of total lipids is represented in last line entry (Lipids).

TABLE 6

Cholesterol Content of Newly Hatched Chick Tissues from Eggs Enriched with n-3 or n-6 Fatty Acids[a]

Tissue	FXC	CAC	SBC	SFC
Plasma	375.00 ± 1.15[c]	378.00 ± 2.45[c]	390.49 ± 2.34[b]	371.00 ± 4.79[c]
Liver	51.11 ± 2.05[c]	60.40 ± 1.61[b]	62.12 ± 1.00[b]	63.55 ± 3.49[b]
Heart	3.29 ± 0.13	3.06 ± 0.35	3.36 ± 0.20	3.02 ± 0.17
Brain	7.69 ± 0.45	7.51 ± 0.33	8.29 ± 0.78	8.25 ± 0.88

[a]Values are presented in mg/g of tissue as mean ± SEM, n = 5. In first line entry (Plasma), mg/dL is represented. Superscripts b–c: means in a row without common superscript differ significantly. FXC, CAC, SBC and SFC represent chicks hatched from hens fed diets containing ground flax, canola, soybean or sunflower seeds, respectively.

chicks. Except for the FXC chicks, the level of 20:4n-6 in brain was not influenced by the yolk fatty acid composition. The level of MUFA in the brain lipids was not affected by yolk fatty acid composition (data not shown).

The chicks hatched from FE eggs enriched with n-3 fatty acids significantly reduced liver cholesterol levels (Table 6). Cholesterol levels in the heart, brain and plasma were not affected by the yolk fatty acid composition.

DISCUSSION

The present study indicates that the presence of n-3 or n-6 fatty acids in the laying hen diet can enrich the egg yolk lipids and further the tissues of the hatched chicks with n-3 and n-6 fatty acids. The increased supply of dietary 18:3n-3 tended to increase the levels of the long-chain n-3 fatty acids such as EPA, DPA, DHA associated with a corresponding reduction in the level of 20:4n-6 in the egg yolk.

The major n-3 fatty acid in liver and brain was DHA. The low LNA and higher DHA levels in liver of n-3 fatty acid enriched FXC and CAC compared to SFC indicate the importance of liver in supplying DHA to the developing brain. Similar levels of DHA in the liver tissue of FXC and CAC chicks further support this observation because the brain of the FXC and CAC chicks had similar DHA levels. Moreover, DHA was a major fatty acid in the chick plasma. Liver and plasma have been shown to provide DHA to the developing brain in rat pups when the maternal diet was enriched with 18:3n-3 (21). Despite the changes in the fatty acid composition of the brain tissue, the total PUFA content (n-6 plus n-3) remained constant in this tissue. Similar observations have been reported for other species, such as the rat (22,23,2) and pig (24).

The chicks hatched from eggs enriched with n-3 fatty acids (FE) reduced plasma and liver cholesterol and plasma total lipids. In contrast, chicks hatched from eggs enriched with n-6 fatty acids showed a similar reduction in plasma cholesterol with an accumulation of cholesterol in the liver. However, this reduction was not evident in the chicks hatched from SBE eggs which might be due to the lower levels of n-3 fatty acids in the SBE eggs. Thus, n-6 and n-3 fatty acids in egg yolk affected cholesterol metabolism in the developing progeny differently. The n-6 fatty acids caused a redistribution of cholesterol from the plasma to the liver pool. Diets rich in n-6 fatty acids have been reported to increase cholesterol esterification. This

increased esterification further increases the capacity of hepatic cells to take up more free cholesterol from circulating plasma (16,25). The rate-limiting step in cholesterol synthesis is 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) (26). Haave *et al.* (27) reported an increased activity of this enzyme in the fetal liver of rat pups when the dam's diet was high in n-6 fatty acids. Differential effects of n-3 and n-6 fatty acids causing redistribution of cholesterol have been reported when rats were fed egg yolks enriched with n-3 or n-6 fatty acids (28) or linseed and sunflower oil (16). However, the enrichment of n-3 and n-6 fatty acid in the egg and their effect on the developing chick have not been reported.

In summary, the type of fat fed to laying hens can affect the fatty acid composition of the yolk and also the fatty acid and cholesterol content of the developing progeny. Whether changes in fatty acid accretion during development due to alteration of yolk fatty acid composition may cause long-term changes in cholesterol metabolism in the chick requires further study. In view of the significant impact of the maternal diet on fetal accretion of PUFA, it will be important to assess the net synthesis of these fatty acids and the appropriate amount of precursor for optimal tissue accretion. The omega-3 fatty acid enriched egg and the newly hatched chick model could be used to study the transfer, uptake and synthesis of polyunsaturated fatty acids during development.

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Effect of Dietary Linoleic Acid Content on the Distribution of Triacylglycerol Molecular Species in Rat Adipose Tissue

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The present study examined the effect of varying dietary linoleate intake (0.01, 0.24, 2.4, 24, 80 or 160 g/kg diet) for 24 weeks on the distribution of triacylglycerol (TG) molecular species in rat epididymal adipose tissue. Adipose TG fractions were purified by thin-layer chromatography and separated into different molecular species by reverse-phase high-performance liquid chromatography. The identification of TG species was based on fatty acid composition, retention time and the theoretical carbon number. When the dietary 18:2n-6 content was equal to or less than 24 g/kg, no significant amounts of n-6 fatty acids (mainly 18:2n-6) were observed in adipose tissue TG despite the fact that the levels of 20:4n-6 in liver phospholipids increased significantly. There were 12 major molecular species in adipose tissue when the dietary 18:2n-6 content was less than 2.4 g/kg. When the dietary 18:2n-6 content reached 24 g/kg, an additional six TG species containing one, two or three molecules of 18:2n-6 were observed. The levels of TG molecules containing two or three 18:2n-6 residues were further increased when the diet contained very large amounts of linoleic acid (160 g/kg). Conversely, those TG species containing only one 18:2n-6 residue became less abundant. It is suggested that the accumulation of these linoleate-rich TG molecular species in adipose tissue, particularly di- and trilinoleoyl containing TG, is the result of an adequate or an excessive intake of linoleic acid.
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Adipose tissue is the most important extrahepatic tissue regulating *in vivo* lipid metabolism. This tissue is rich in triacylglycerols (TG), whose fatty acid composition is modulated by *in situ de novo* synthesis and the influx of fatty acids from the diet or from the *de novo* synthesis in the liver (1–3). During early development, adipose tissue is very active in fatty acid synthesis (4–6). However, as animals grow older, the amount of fatty acid synthesis *in situ* declines, while the deposition of lipid from exogenous fat increases (7–10). As a result, dietary fat intake modulates the fatty acid composition of adipose tissue in mature animals.

In recent years, it has become well established that high levels of plasma cholesterol correlate closely with a high risk of coronary heart disease, and that dietary polyunsaturated fatty acids (mainly linoleic acid, 18:2n-6) can lower plasma cholesterol levels (11). As adipose tissue fatty acid composition reflects the history of dietary linoleic acid intake, it has been suggested that low adipose tissue linoleic acid is a risk factor in coronary heart disease (12,13). Previously, such assessments were based on overall linoleic acid content. The exact distribution of linoleic acid in TG species could not be routinely examined because of a lack of simple and effective techniques for the separation of TG species. Using $AgNO_3$-thin layer chromatography ($AgNO_3$-TLC) followed by gas-liquid chromatography (GLC), Bugaut (14) has examined the adipose tissue TG molecular species in rats fed a diet containing coconut oil. In the present paper we examined whether changes in dietary linoleic acid content would affect the distribution of linoleic acid containing TG in adipose tissue. The separation of TG species was carried out using a simple reverse-phase high-performance liquid chromatography (HPLC) method with a new isocratic solvent system (isopropranol/acetonitrile, 35:65, vol/vol).

MATERIALS AND METHODS

Weanling male Sprague-Dawley rats (3-wk-old) were purchased from the Charles River Breeding Laboratories (St. Constant, Quebec, Canada). Animals were randomly divided into six groups of six each and maintained *ad libitum* on a fat-free semi-purified diet (Teklad Test Diet, Madison, WI) supplemented with 20% (by weight) of fat containing 0.005, 0.12, 1.2, 12, 40 and 80% of linoleic acid (equivalent to 0.01, 0.24, 2.4, 24, 80 and 160 g/kg in the diet). The fat was prepared by mixing different proportions of safflower oil (containing 80% 18:2n-6) and hydrogenated coconut oil (containing no significant amount of n-6 fatty acids). The detailed composition of the fat-free diet has been described previously (15). Briefly, the diet contained 20% vitamin-free casein, 69.7% sucrose, 5% cellulose, 3.5% mineral mix (AIN-76), 1% vitamin mix, 0.5% calcium carbonate and 0.3% DL-methionine. After 24 weeks on the respective diet, all animals were killed in the fed state by exsanguination under light diethyl ether anaesthesia. Livers and the epididymal fat pads were excised, rinsed in cold saline, blotted, weighed, and frozen at −20°C.

Total tissue lipids were extracted, and the TG fraction was purified by TLC using hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol) as the mobile phase. The purified adipose tissue TG fraction (1–2 mg dissolved in 5–10 μL chloroform) was then fractionated, according to the number of double bonds and acyl carbons, into different molecular species by HPLC using a Beckman System Gold programmable solvent module 126 (Beckman Instruments, Palo Alto, CA). Two identical reverse-phase columns, Supelcosil LC-18 (5 μ, 250 mm × 4.6 mm i.d.; Supelco, Bellefonte, PA), maintained at 30°C in a programmable column oven, were used as the stationary phase, while isopropanol/acetonitrile (35:65, vol/vol) was used isocratically as the mobile phase (flow rate 2 mL/min). For preparative analysis, the eluting TG components were detected using a UV detector (Model 166, Beckman) at 210 nm. The presence of isopropanol (which has a UV cut-off value at 210 nm) in the mobile phase caused some UV absorption (approximately 0.34 units). However, the background value was automatically offset and only the net absorbance changes were shown in the computer monitor. The eluting TG components monitored with UV detector were then collected. A known amount of triheptadecanoin

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Abbreviations: GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; L, linoleic acid (18:2n-6); O, oleic acid (18:1n-9); P, palmitic acid (16:0); PL, phospholipids; Po, palmitoleic acid (16:1n-7); S, stearic acid (18:0); TG, triacylglycerols; TLC, thin-layer chromatography.

(tri-17:0) was added as an internal standard to each collection prior to methylation and GLC analysis. Identification of TG species was achieved by examining their fatty acid composition analyzed on a fused silica capillary column (Supelcomega, 50 m × 0.25 mm i.d.; Supelco) in a Hewlett-Packard Model 5890 gas-liquid chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector (16). For quantitation, the eluting TG components were detected using a mass (light scattering) detector (ACS model 740/14, Applied Chromatography System Ltd., Macclesfield, England). The settings on the mass detector were those described previously (17). The output signal integration and data analysis were performed on an IBM PS/2 computer (model 50) using Gold Software (version 3.1, Beckman). The response of the mass detector to different molecular species was compared to the value determined by GLC analysis using the added internal standard (tri-17:0), and the correction factor for each molecular species was then derived. A computer program described by Merritt *et al.* (18) was modified and used to determine the TG molecular species. The computation was based on random probability calculations (19,20) using the fatty acid composition of the purified TG fraction, and on the retention time, which has a direct logarithmic relation to the theoretical carbon number (21). It should be noted that the present system could not resolve positional TG isomers having the same acyl groups but in different positions (*e.g.*, OOL, OLO and LOO). Peaks identified could represent a mixture of the corresponding isomers. One-way analysis of variance was used to assess the significance of differences between groups fed different levels of linoleic acid.

RESULTS

Figure 1 shows the changes of two major n-6 fatty acids, 18:2n-6 (panel A) and 20:4n-6 (panel B), in liver and adipose tissue TG and phospholipids (PL) in response to different dietary linoleate levels. In liver and adipose tissue PL, 18:2n-6 constituted a small proportion of total fatty acids. However, the levels of 18:2n-6 were increased as the dietary linoleate content increased. The levels of 20:4n-6 were similarly increased in both tissues (Fig. 1, panel B). However, the magnitude of change was significantly lower in adipose tissue PL than in liver PL. In adipose tissue and liver TG, the levels of 18:2n-6 also were very low and were not significantly affected by increasing the dietary linoleate content up to 2.4 g/kg. As shown in Figure 1, the levels of 18:2n-6 in animals fed 2.4 g/kg (as compared with those fed either 0.01 or 0.24 g/kg linoleic acid) were increased by only a small fraction, despite a 240- and 10-fold increase in the dietary supply of linoleic acid. When the dietary linoleate content reached 24 g/kg diet, the levels of 18:2n-6 in adipose tissue and liver TG were increased significantly. Similarly, the levels of 20:4n-6 in liver and adipose tissue TG, undetectable when dietary linoleate content was less than 24 g/kg, were increased slightly in liver TG but not in adipose tissue TG, when dietary linoleate exceeded 24 g/kg (Fig. 1, panel B). The detailed fatty acid composition of adipose tissue TG from animals fed diets supplemented with linoleate at three different levels (0.01, 24 and 160 g/kg) is shown in Table 1.

Figure 2 shows two typical HPLC chromatograms of adipose tissue TG species from rats fed a diet containing

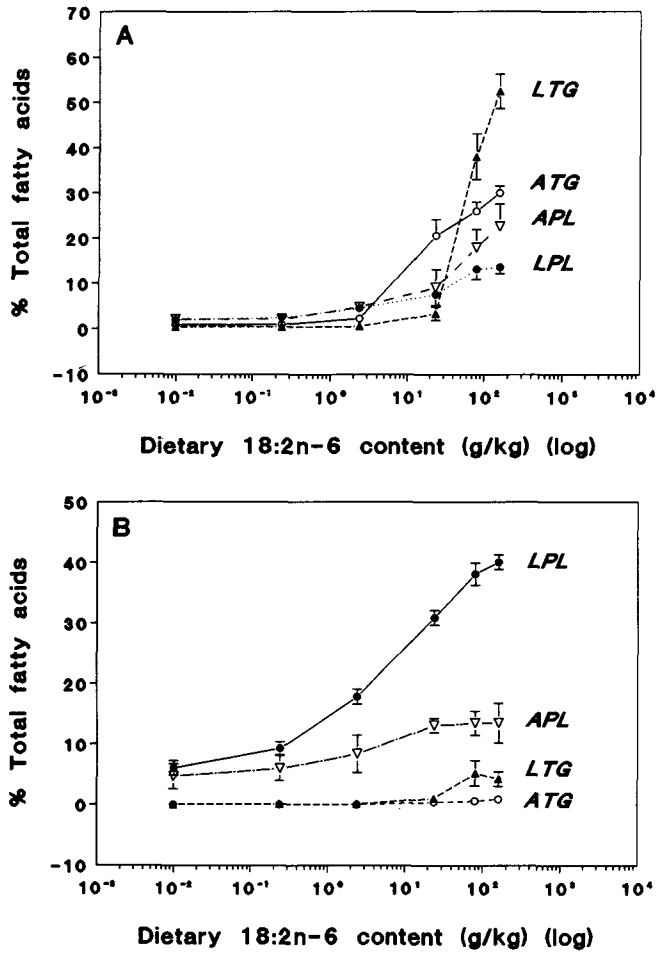

FIG. 1. The levels (mol% of total fatty acids) of 18:2n-6 (panel A) and 20:4n-6 (panel B) in liver phospholipids (LPL), liver triacylglycerols (LTG), and epididymal fat phospholipids (APL) and triacylglycerols (ATG) of rats fed a fat-free diet supplemented with linoleic acid at a level of 0.01, 0.24, 2.4, 24, 80 or 160 g/kg (in log) for 24 weeks (mean ± SD of six animals).

TABLE 1

Triacylglycerol Fatty Acid Composition (mol%) in Epididymal Fat of Rats Fed a Semi-Synthetic Diet Supplemented with 0, 24 and 160 g/kg of Linoleic Acid in the Diet for 24 Weeks[a]

Fatty acids	Dietary 18:2n-6 content (g/kg)		
	0.01	24	160
14:0	2.8 ± 0.4[a]	1.4 ± 0.1[b]	1.2 ± 0.1[b]
16:0	22.3 ± 1.0	24.1 ± 2.1	20.1 ± 2.6
16:1n-7	15.6 ± 2.1[a]	12.2 ± 2.2[b]	10.2 ± 1.9[b]
18:0	1.9 ± 0.2	2.0 ± 0.4	2.0 ± 0.1
18:1n-9	52.0 ± 2.2[a]	36.5 ± 2.1[b]	33.3 ± 3.4[b]
18:2n-6	0.9 ± 0.2[a]	20.6 ± 3.6[b]	30.1 ± 4.5[b]
20:3n-6	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.2 ± 0.0[b]
20:4n-6	0.0 ± 0.0[a]	0.4 ± 0.1[b]	0.5 ± 0.1[b]
Others[b]	4.5 ± 0.6[a]	2.8 ± 0.3[b]	2.4 ± 1.1[b]

[a]The values (means ± SD of six animals) with different superscripts (a,b) are significantly different at $P < 0.05$.
[b]Others include 18:1n-7, 20:1 and 20:3n-9.

linoleic acid at a level of 0.01 and 24 g/kg. It is evident that the separation of various TG species based on theoretical carbon number and degree of unsaturation was satisfactory. The random and experimental distributions

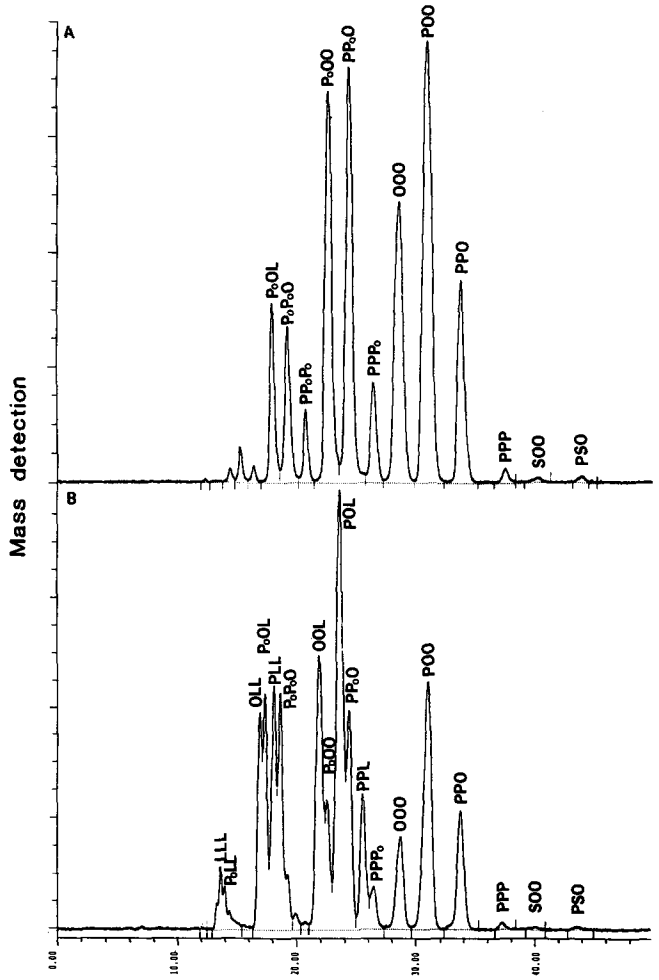

FIG. 2. Typical HPLC chromatograms of adipose tissue triacylglycerol molecular species from rats fed a diet containing linoleic acid at a level of 0.01 g/kg (panel A) or 24 g/kg (panel B).

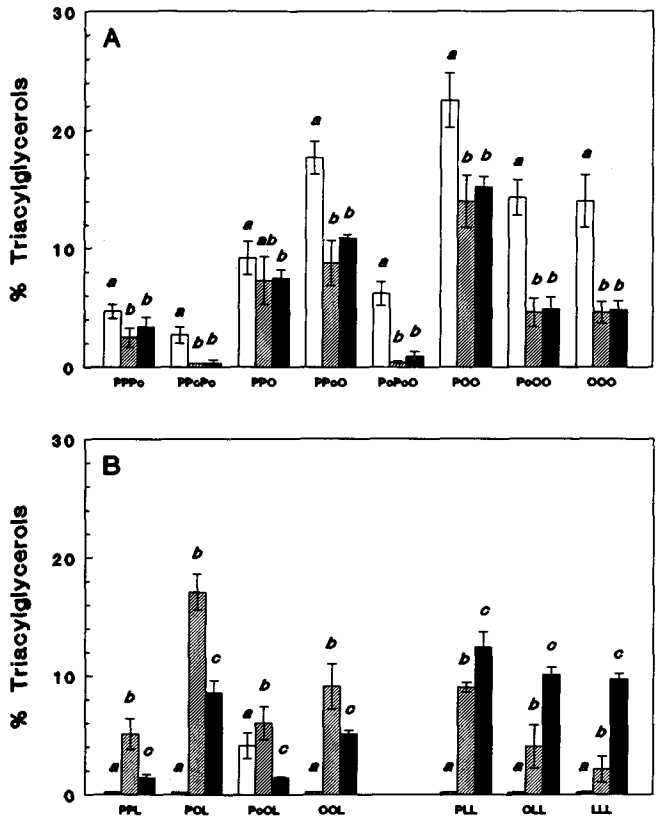

FIG. 3. Distribution (mol%) of linoleoyl deficient (panel A) or linoleoyl containing (panel B) triacylglycerol molecular species in epididymal fat pads from rats fed a diet supplemented with linoleic acid at a level of 0.01 (white bars), 24 (hatched bars) or 160 (black bars) g/kg for 24 weeks (mean ± SD of six animals). ^{a,b,c}Data with different superscripts are significantly different at $P < 0.05$. Abbreviations: P, palmitic, 16:0; Po, palmitoleic, 16:1n-7; S, stearic, 18:0; O, oleic, 18:1n-9; L, linoleic, 18:2n-6.

of TG molecular species in epididymal adipose tissue from rats fed low linoleic acid (0.01 g/kg diet) or linoleic acid-supplemented diet (24 and 160 g/kg diet) for 24 weeks are shown in Table 2. The distribution of major linoleic acid-deficient (panel A) and linoleic acid-containing (panel B) TG molecular species in epididymal adipose tissue are shown in Figure 3. In animals fed linoleic acid-sufficient diets (24 and 160 g/kg) as compared with those receiving lower amounts (0.01, 0.24 and 2.4 g/kg), the levels of linoleic acid containing TG molecular species, such as LLL, OLL, PLL, OOL, POL, and PPL, were significantly increased, whereas the levels of PoPoO, PoOO, PPoO, PPPo, OOO and POO were decreased. There were no significant differences between the overall levels of linoleoyl containing TG molecular species (52.4 *vs.* 49.6% of total TG) in rats receiving 24 and 160 g/kg of linoleic acid in the diet. However, the higher the dietary linoleic acid content, the greater the increase in the levels of TG species containing two or three molecules of 18:2n-6 (*e.g.*, PLL, OLL and LLL). This increase was at the expense of the levels of mono-linoleoyl containing TG species (*e.g.*, PPL, POL, and OOL), which were lower in adipose tissue from rats fed 160 g/kg than those fed 24 g/kg linoleic acid.

DISCUSSION

The results in Figure 1 show that the levels of 20:4n-6 in both adipose tissue and liver PL were increased as dietary linoleate content increased. On the other hand, the levels of 20:4n-6 in liver and adipose TG were very low when dietary linoleate content was below 2.4 g/kg. This suggests that at this level (2.4 g/kg) most of the linoleic acid taken up by the animal was metabolized and used in the synthesis of membrane PL (3,22–24). When dietary linoleate content exceeded 24 g/kg, the increased 18:2n-6 levels in both liver and adipose TG indicated that the dietary linoleate was likely to have exceeded the minimum requirement for PL synthesis, and some was incorporated into TG for storage. Thus, based on the present results, the minimum linoleate requirement of the animals was estimated to be 0.5–5% of total calories. This is in agreement with a previous report in which Holman (25) estimated the requirement to be 1–2% of total calories.

The use of HPLC to determine TG species in this study shows the unequivocal changes in mono- and di- or trilinoleoyl containing TG species (Fig. 3). When dietary 18:2n-6 content was below 24 g/kg (0.1, 0.24 or 2.4 g/kg), adipose tissue predominantly contained the non-linoleoyl containing TG, and only trace amounts of the linoleoyl containing TG. The latter became significant only when

TABLE 2

Experimental and Random Distribution (mol%) of Triacylglycerol Molecular Species in Epididymal Fat from Rats Fed a Semi-Synthetic Diet Supplemented with 0.01, 24 and 160 g/kg of Linoleic Acid (mean ± SD of six animals) for 24 Weeks[a]

TG species	Dietary 18:2n-6 conent (g/kg)					
	0.01 g/kg		24 g/kg		160 g/kg	
	Random	Experiment	Random	Experiment	Random	Experiment
LLL	—	—	0.9	2.1 ± 1.1	2.7	9.7 ± 0.5
PoLL	—	—	1.6	—	2.8	—
PoPoL	—	—	0.9	—	0.9	—
OLL	—	—	4.7	4.0 ± 1.8	9.1	10.1 ± 0.6
PoOL	—	4.1 ± 1.1	5.5	6.0 ± 1.4	6.1	1.4 ± 0.1
PLL	—	—	3.1	8.0 ± 0.4	5.5	12.4 ± 1.3
PoPoO	3.8	6.2 ± 1.0	1.6	0.4 ± 0.1	1.0	—
POoL	—	—	3.6	—	3.7	0.9 ± 0.4
MOL	—	—	0.6	—	0.7	—
PPoPo	1.6	2.7 ± 0.7	1.1	0.3 ± 0.0	0.6	1.0 ± 0.5
OOL	0.7	—	8.3	9.1 ± 1.9	10.0	5.1 ± 0.3
PoOO	12.7	14.3 ± 1.5	4.9	4.6 ± 1.2	3.4	4.9 ± 1.0
POL	0.6	—	10.9	17.1 ± 1.5	12.1	8.6 ± 1.0
PPoO	10.9	17.7 ± 1.4	6.4	8.8 ± 1.9	4.1	10.9 ± 0.3
MOO	2.3	—	0.6	—	0.5	—
PPL	—	—	3.6	5.1 ± 1.3	3.7	1.4 ± 0.3
MPO	1.9	—	0.8	—	0.5	—
PPPo	2.3	4.7 ± 0.6	2.1	2.5 ± 0.8	1.2	3.4 ± 0.8
OOO	14.0	14.0 ± 2.2	4.9	4.6 ± 0.9	3.7	4.8 ± 0.8
SOL	—	—	0.9	—	1.2	—
POO	18.1	22.5 ± 2.3	9.6	14.0 ± 2.2	6.7	15.2 ± 0.9
PoSO	0.9	—	0.5	—	0.5	—
PSL	—	—	0.6	—	0.7	—
PPO	7.8	9.2 ± 1.4	6.4	7.3 ± 2.0	4.0	7.5 ± 0.7
PPP	1.1	0.7 ± 0.6	1.4	0.8 ± 0.6	0.8	0.1 ± 0.1
SOO	1.5	0.4 ± 0.2	0.8	0.3 ± 0.1	0.7	0.2 ± 0.1
PSO	1.3	0.3 ± 0.1	1.1	0.3 ± 0.1	0.8	0.2 ± 0.2
Others	18.5	3.2	12.6	4.7	12.3	2.2

[a]Fatty acid abbreviations: M, myristic; P, palmitic; Po, palmitoleic; S, stearic; O, oleic; L, linoleic; X, non-linoleic. Theoretical distribution (>0.5%) of triacylglycerol molecular species was calculated from total fatty acid composition (shown in Table 1) based on 1,2,3-random.

dietary linoleate content reached 24 g/kg. Since increasing the levels of linoleoyl containing TG reduced only a portion of non-linoleoyl containing TG in adipose tissue, this suggests that the increased influx of dietary linoleate might have partially suppressed endogenous fatty acid synthesis. There is ample evidence to demonstrate that dietary 18:2n-6 suppresses endogenous fatty acid synthesis in liver (26,27), but not in adipose tissue (28,29), and that feeding a linoleic acid-rich diet limited the contribution of *de novo* synthesized fatty acids from liver to the adipose tissue fatty acid store (see review, ref. 30). In the present study, we observed that the proportion of non-linoleoyl containing TG in adipose tissue was reduced to the same extent in animals given diets containing either moderately or extremely high levels (24 or 160 g/kg) of linoleic acid (Fig. 3, panel A). This finding suggests that the *in situ* formation of non-linoleoyl containing TG in adipose tissue was not affected by the dietary linoleate content. Thus, a decrease in the level of non-linoleoyl containing TG was probably the result of a reduced influx of *de novo* synthesized fatty acids from liver in conjunction with a significant increase in the formation of linoleoyl containing TG in adipose tissue. Nelson *et al.* (31) have shown that dietary linoleate is rapidly incorporated into adipose tissue TG in animals fed a linoleate-rich diet. Comparison between animals fed diets containing low and moderate levels (2.4 and 24 g/kg) of linoleic acid showed that the level of 18:2n-6 in adipose tissue TG was increased in parallel with the dietary linoleate content. However, when linoleic acid was increased from 24 to 160 g/kg (> six-fold increase), the level of 18:2n-6 in adipose tissue TG was increased by only 45% (Table 1). In other words, increases in the level of 18:2n-6 in adipose tissue are less pronounced once the linoleate content in the diet exceeds 24 g/kg, or equivalent to 5% of total calories. This value is therefore greater than the minimum dietary requirement. As dietary linoleate content increased from moderate to high levels (from 24 to 160 g/kg), the levels of di- and trilinoleoyl containing TG in adipose tissue were increased two-fold (32.2 *vs* 15.1%), whereas those of monolinoleoyl containing TG were decreased concomitantly two-fold (37.3 *vs.* 17.4%). This finding suggests that the additional linoleic acid molecules might have replaced fatty acids, such as 16:0, 16:1 or 18:1 in the monolinoleoyl containing TG, and converted them to di- or trilinoleoyl containing TG. Thus, the ratio of poly- to monolinoleoyl containing TG molecular species may be a useful indicator for assessing the level of dietary linoleate intake and its adequacy. Moreover, in comparison with the theoretical distribution calculated on basis of 1,2,3-random linoleate distribution, the observed levels of polylinoleoyl containing TG were increased (Table 2). It appears that upon an

increase in supply, linoleic acid was incorporated more readily into TG molecules already containing one or two 18:2n-6 residues than into those containing no linoleic acid.

In conclusion, we observed that the proportion of linoleoyl containing TG in adipose tissue increased only when the dietary linoleate content reached a certain level (24 g/kg). A further increase in dietary linoleic acid had only a moderate effect on the overall adipose tissue fatty acid composition, but it significantly modified the distribution of linoleic acid among adipose tissue TG molecular species; the proportion of monolinoleoyl containing TG species was decreased, whereas that of polylinoleoyl containing TG species was increased.

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Site-Specific Differences in the Fatty Acid Composition of Human Adipose Tissue

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The fatty acid composition of triacylglycerols from fifteen distinct adipose depots taken from each of seven adult male human subjects was compared. Oleic, palmitic, linoleic, stearic, myristic, palmitoleic and vaccenic acids accounted for more than 90% of the triacylglycerol fatty acids in all sites from all subjects; a number of other fatty acids were also identified and quantified. There were large differences in the average fatty acid composition between individual subjects. There were no site-specific differences in the proportions of myristic (3.8–4.7% of triacylglycerol fatty acids), palmitic (23–29%), linoleic (6.7–9.8%) or vaccenic (4.1–4.7%) acids or in the proportions of any of the less abundant fatty acids. There were some significant site-specific differences in the proportions of palmitoleic, oleic and stearic acids. The calf depot contained more palmitoleic acid (6.41 ± 1.09%) than the trapezius (3.12 ± 0.55%), perirenal (3.59 ± 0.50%) and mesenteric (3.70 ± 0.43%) depots, more oleic acid (42.13 ± 1.27%) than the trapezius (36.03 ± 2.18%), perirenal (36.50 ± 1.56%) and breast (37.13 ± 1.55%) depots and less stearic acid (5.18 ± 0.89%) than the trapezius (8.57 ± 0.97%), perirenal (8.49 ± 0.75%), mesenteric (7.87 ± 0.42%), breast (8.02 ± 0.75%) and clavicular (8.34 ± 0.78%) depots. The buttock depot contained less stearic acid (6.06 ± 0.65%) than the perirenal, mesenteric and clavicular depots, while the anterior thigh depot contained less stearic acid (6.07 ± 0.70%) than the perirenal depot. These findings indicate that, while most human adipose depots differ little in fatty acid composition, some sites, in particular the calf, perirenal, trapezius and mesenteric depots, have site-specific properties.
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A number of studies have documented differences between human adipose depots with respect to adipocyte size (1–6) and metabolism (1–8), including lipoprotein lipase activity (1), sensitivity of lipolysis to catecholamines and to starvation (2,7,8), sensitivity to the antilipolytic effect of insulin (3), high density lipoprotein metabolism (5), glycolytic capacity (6) and adenylate cyclase activity and its sensitivity to adrenaline (7). Despite these structural and metabolic differences between adipose depots, it is generally assumed that the fatty acid composition of all adipose tissue within an individual is the same (9). Several studies have compared the fatty acid composition of different human adipose depots (10–15) and, although some of these studies did not detect any site-specific differences, some consistent differences have been reported. The adipose depots investigated in these studies were chosen on the basis of surgical accessibility. As a result, previous studies have compared a limited number of sites, usually two (11,14), three (10,12,15) or four (12). Furthermore, some of the earlier studies compared different depots from only a small number of subjects, in some cases from a single individual (10,12). To determine the true extent of site-specific differences in fatty acid composition, a large number of depots from a number of individuals should be compared. In this study we investigated the fatty acid composition of fifteen distinct adipose depots from seven subjects. The adipose depots examined included those studied previously (10–15) and those believed, on the basis of their anatomical relations with other tissues (see ref. 16), to be homologous with depots that are frequently studied in laboratory rodents (17–20) and other mammals (21–25). To our knowledge, this investigation of site-specific differences in human adipose tissue fatty acid composition is the most extensive yet performed.

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Abbreviations: BSTFA, *N,O*-bis-(trimethylsilyl)trifluoroacetamide; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry.

MATERIALS AND METHODS

Materials. Solvents were obtained from Fisons Scientific Apparatus (Loughborough, Leics., U.K.) and were redistilled before use. *N,O-bis*-(Trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). An ethereal solution of diazomethane was prepared from Diazald (Aldrich Chemical Co., Gillingham, Dorset, U.K.).

Source of adipose tissue. Adipose tissue samples were taken at *post mortem* examination from seven fresh, adult male cadavers for whom death had been sudden and/or accidental (for example, road traffic accident, drowning). The seven subjects were aged 31, 35, 27, 25, 46, 63 and 47 years, respectively (mean ± SD age 39 ± 13 years). All subjects lived in Southwest England. Subjects who had died from chronic illnesses were excluded from the study. The samples were collected within 24 h of death and were individually sealed and stored at −20°C until analysis. The adipose depots sampled included eight superficial (buttock, breast, clavicular, abdominal wall, anterior thigh, medial thigh, posterior arm, anterior arm), three intermuscular (popliteal, under the trapezius muscle, calf), three intraabdominal (perirenal, mesenteric, omental) and one intrathoracic (pericardial) sites. The calf depot, although not anatomically intermuscular, is included here as an intermuscular site because it is homologous with an intermuscular depot in other mammals (16).

Lipid extraction and fatty acid analysis. Total lipid was extracted using chloroform/methanol (2:1 vol/vol) as described by Folch *et al.* (26). Triacylglycerols were separated from phospholipids by chromatography through a column of activated silicic acid prewashed with chloroform. Triacylglycerols were eluted from the column with chloroform. Triacylglycerol fatty acids were obtained by saponification with methanolic 0.5 M KOH for 120 min at 70°C and were extracted into ethyl acetate. After evaporation to dryness, fatty acid methyl esters were prepared by reaction with an excess of diazomethane in diethyl ether (27).

TABLE 1

Average Fatty Acid Composition (moles %) of All Adipose Depots from Seven Human Subjects

Subject	Percentage of total fatty acid[a]						
	14:0	16:1n-7	16:0	18:2n-6	18:1n-9	18:1n-7	18:0
1	3.36 ± 0.11^{cdefgh}	3.55 ± 0.40^{ceh}	21.94 ± 0.58^{efgh}	12.39 ± 0.18^{cdefgh}	41.71 ± 0.81^{dfgh}	4.66 ± 0.16^{d}	6.68 ± 0.51^{cf}
2	2.96 ± 0.08^{bdefg}	5.69 ± 0.25^{bdfg}	21.70 ± 0.35^{defgh}	10.76 ± 0.22^{bdefgh}	42.85 ± 0.46^{dfgh}	4.39 ± 0.08^{dg}	4.69 ± 0.29^{bdefgh}
3	4.50 ± 0.12^{bcefg}	3.04 ± 0.08^{ceh}	23.52 ± 0.79^{cfgh}	16.08 ± 0.38^{bcefgh}	35.93 ± 0.78^{bceg}	2.70 ± 0.06^{bcefgh}	7.22 ± 0.29^{cf}
4	4.17 ± 0.07^{bcdfg}	5.96 ± 0.22^{bdfg}	24.10 ± 0.31^{bcfgh}	5.44 ± 0.46^{bcdg}	42.59 ± 0.27^{dfgh}	4.58 ± 0.11^{d}	7.29 ± 0.20^{cf}
5	5.65 ± 0.16^{bcdeg}	2.83 ± 0.28^{ceh}	32.30 ± 0.95^{bcdeg}	6.35 ± 0.37^{bcd}	34.36 ± 0.86^{bceg}	4.59 ± 0.26^{d}	9.85 ± 0.47^{bcdegh}
6	5.01 ± 0.11^{bcdef}	3.56 ± 0.32^{ceh}	28.55 ± 1.17^{bcdef}	7.07 ± 0.24^{bcdeh}	38.83 ± 1.08^{bcdef}	5.05 ± 0.21^{cd}	7.78 ± 0.43^{cf}
7	5.39 ± 0.18^{bcde}	5.39 ± 0.47^{bdfg}	30.78 ± 1.18^{bcde}	6.27 ± 0.15^{bcdg}	36.60 ± 1.02^{bce}	4.69 ± 0.15^{d}	7.35 ± 0.50^{cf}

[a]Data are mean ± SE from 14 (subjects 1, 3 and 6) or 15 (subjects 2, 4, 5 and 7) different anatomical sites. Statistical significance ($P < 0.05$) is indicated by ^{b}*vs.* subject 1, ^{c}*vs.* subject 2, ^{d}*vs.* subject 3, ^{e}*vs.* subject 4, ^{f}*vs.* subject 5, ^{g}*vs.* subject 6 and ^{h}*vs.* subject 7.

TABLE 2

Fatty Acid Composition (moles %) of Fifteen Adipose Depots from Human Subjects

Adipose depot	Percentage of total fatty acid[a]						
	14:0	16:1n-7	16:0	18:2n-6	18:1n-9	18:1n-7	18:0
Superficial							
Buttock (n = 7)	4.11 ± 0.34	4.84 ± 0.58	25.22 ± 1.59	8.90 ± 1.40	40.61 ± 1.22	4.26 ± 0.31	6.06 ± 0.65^{deg}
Breast (n = 7)	4.51 ± 0.43	3.64 ± 0.75	26.99 ± 2.25	9.76 ± 1.56	37.13 ± 1.55^{b}	4.50 ± 0.33	8.02 ± 0.75^{b}
Clavicular (n = 7)	4.69 ± 0.47	3.77 ± 0.66	26.01 ± 1.25	9.20 ± 1.36	38.76 ± 1.08	4.40 ± 0.36	8.34 ± 0.78^{bh}
Abdominal wall (n = 7)	4.49 ± 0.46	3.94 ± 0.59	26.59 ± 2.16	9.25 ± 1.99	38.89 ± 1.85	4.27 ± 0.31	7.40 ± 0.88
Anterior thigh (n = 7)	4.18 ± 0.42	4.31 ± 0.59	25.70 ± 2.11	9.51 ± 1.54	39.91 ± 1.47	4.14 ± 0.24	6.07 ± 0.70^{d}
Medial thigh (n = 7)	4.22 ± 0.37	4.89 ± 0.67	25.74 ± 2.08	9.02 ± 1.71	39.80 ± 1.80	4.64 ± 0.33	6.62 ± 0.81
Posterior arm (n = 7)	4.41 ± 0.43	4.46 ± 0.53	26.73 ± 1.95	9.24 ± 1.51	39.30 ± 1.61	4.20 ± 0.34	6.96 ± 0.71
Anterior arm (n = 7)	4.54 ± 0.32	4.16 ± 0.45	27.53 ± 1.42	9.11 ± 1.79	39.34 ± 1.50	4.24 ± 0.41	7.39 ± 0.61
Intermuscular							
Popliteal (n = 7)	4.36 ± 0.41	4.26 ± 0.58	25.54 ± 1.91	9.53 ± 1.55	39.17 ± 1.59	4.54 ± 0.33	6.96 ± 0.58
Trapezius (n = 6)	4.70 ± 0.53	3.12 ± 0.55^{b}	28.38 ± 2.74	9.55 ± 1.68	36.03 ± 2.18^{b}	4.20 ± 0.40	8.57 ± 0.97^{b}
Calf (n = 6)	3.78 ± 0.41	6.41 ± 1.09^{cde}	23.47 ± 1.85	8.98 ± 1.74	42.13 ± 1.27^{cdf}	4.27 ± 0.33	5.18 ± 0.89^{cdefg}
Intraabdominal and intrathoracic							
Perirenal (n = 7)	4.67 ± 0.40	3.59 ± 0.50^{b}	27.43 ± 2.38	8.84 ± 1.70	36.50 ± 1.56^{b}	4.59 ± 0.51	8.49 ± 0.75^{bhi}
Mesenteric (n = 7)	4.61 ± 0.34	3.70 ± 0.43^{b}	25.26 ± 1.11	9.44 ± 1.12	38.37 ± 1.21	4.20 ± 0.35	7.87 ± 0.42^{bh}
Omental (n = 7)	4.60 ± 0.40	4.44 ± 0.62	23.91 ± 2.36	9.18 ± 1.30	40.50 ± 2.69	4.60 ± 0.54	7.38 ± 0.80
Pericardial (n = 5)	4.46 ± 0.67	4.92 ± 0.83	29.52 ± 3.01	6.72 ± 0.94	37.10 ± 2.38	4.70 ± 0.46	7.36 ± 1.42

[a]Data are mean ± SE from the indicated number of different subjects. Statistical significance ($P < 0.05$) is indicated by ^{b}*vs.* calf, ^{c}*vs.* trapezius, ^{d}*vs.* perirenal, ^{e}*vs.* mesenteric, ^{f}*vs.* breast, ^{g}*vs.* clavicular, ^{h}*vs.* buttock, ^{i}*vs.* anterior thigh.

Fatty acid methyl esters (dissolved in methyl acetate) were separated by gas chromatography (GC) in a Hewlett-Packard (Hewlett-Packard Ltd., Bracknell, Bucks., U.K.) 5890A gas chromatograph 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 130–380°C at 2°C/min. The separation was recorded with a Servoscribe chart recorder and quantitative data were recorded with a Hewlett-Packard 3390A recording integrator. Fatty acid methyl esters were identified by comparison with standards or by gas chromatography/mass spectrometry (GC/MS) of fatty acid trimethylsilyl or picolinyl esters.

Trimethylsilyl esters of fatty acids were prepared by heating with BSTFA for 10 min at 60°C. Picolinyl esters of fatty acids were prepared by the method of Harvey (28,29). GC/MS data were obtained with a VG (Fisons Instruments, Manchester, U.K.) 70/70F mass spectrometer interfaced to a Varian (Varian Associates, Sunnyvale, CA) 2440 gas chromatograph fitted with a SGE split/splitless injection system operated in the split mode with a split ratio of 10:1. The column was a 25 m × 0.2 mm OV-1 bonded phase fused silica capillary, film thickness 0.33 μm, terminating 10 mm inside the mass spectrometer ion source. Helium at 1 mL/min (determined in the absence of the mass spectrometer vacuum) was used as the carrier gas. The injector, transfer line and ion source temperatures were 300, 300 and 280°C, respectively. The column oven temperature was programmed from 180–350°C at 2°C/min. The accelerating voltage, electron energy, trap current and scan speed used were 4 kV, 70 eV, 1 mA and 1 s/decade, respectively. Spectra were recorded with a VG 11/250 data system.

Data presentation and statistics. All data are presented as mean ± SE for the indicated number of samples. Statistical significance was determined using the two-tailed unpaired Student's *t*-test; a value for *P* of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Fatty acid analysis. Between 25 and 30 fatty acids were positively identified in most samples from each of the subjects. However, seven fatty acids comprised more than 90% of the triacylglycerol fatty acids in all sites from all subjects. These were oleic (18:1n-9), palmitic (16:0), linoleic (18:2n-6), stearic (18:0), myristic (14:0), palmitoleic (16:1n-7) and vaccenic (18:1n-7) acids. The next most abundant fatty acids were lauric (12:0) and eicosamonoenoic (20:1n-9) acids, which comprised 0.3–1.0% and 0.4–1.0%, respectively, of the fatty acids present in the samples. The proportion of arachidonic acid (20:4n-6) was 0.1–0.3%. Most samples also contained quantifiable amounts of 14:1n-5 (0.2–0.5%), 15:0 (0.2–0.7%), 16:1n-8 (0.1–0.4%), 17:1n-8 (0.3–0.8%), 17:0 (0.2–0.7%), 20:1n-6 (0.1–0.3%), 20:0 (0.1–0.4%) and 22:1n-9 (0.1–0.2%) and trace amounts (<0.1% of fatty acids) of 10:0, *i*-15:0, *ai*-15:0, 16:1n-6, *ai*-17:0, 18:1n-5, 19:1n-10, 19:1n-8, 20:2n-7, 20:1n-7 and 22:0. In addition, many of the samples contained an eighteen carbon fatty acid whose structure has not yet been elucidated. Neither α- nor γ-linolenic acids (18:3n-3 and 18:3n-6, respectively) were detected in any of the samples.

Intersubject differences in fatty acid composition. The average proportions of the seven major fatty acids in all sites of each subject are shown in Table 1. There were significant differences in the proportion of each of these fatty acids between several of the subjects (Table 1). The greatest differences were in the proportions of palmitoleic and linoleic acids, which varied by up to three-fold between the subjects.

Site-specific differences in fatty acid composition. The proportions of each of the seven major fatty acids in each adipose depot from all subjects are shown in Table 2. There were no consistent site-specific differences in the proportions of myristic, palmitic, linoleic or vaccenic (Table 2) or in the proportions of any of the less abundant fatty acids (data not shown). There were, however, some significant site-specific differences in the proportions of palmitoleic, oleic and stearic acids (Table 2). These differences are summarized in Table 3.

Fatty acid composition data may be summarized in a variety of ways, such as proportions of saturated, monounsaturated and polyunsaturated fatty acids, as a saturated fatty acid/unsaturated fatty acid ratio or as the index of unsaturation, which takes into account the degree of unsaturation of the fatty acids. The fatty acid composition of each of the adipose depots studied is thus summarized in Table 4; data for all identified fatty acids, not only the seven major fatty acids, were used. No site-specific differences in the overall proportions of saturated or polyunsaturated fatty acids, in the saturated/unsaturated fatty acid ratio or in the index of unsaturation were found. There were, however, some site-specific differences in the proportion of monounsaturated fatty acids: the calf adipose

TABLE 3

Summary of the Site-Specific Differences in Human Adipose Tissue Fatty Acid Composition

Fatty acid	Site-specific differences
Palmitoleic	Calf > Trapezius, Perirenal, Mesenteric
Oleic	Calf > Trapezius, Perirenal, Breast
Stearic	Calf < Trapezius, Perirenal, Mesenteric, Breast, Clavicular Buttock < Perirenal, Mesenteric, Clavicular Anterior thigh < Perirenal

TABLE 4

Summary of the Fatty Acid Composition of Fifteen Adipose Depots from Human Subjects[a]

Adipose depot	% Saturated	% Monounsaturated	% Polyunsaturated	Saturated/Unsaturated	Index of unsaturation
Superficial					
Buttock (n = 7)	38.4 ± 2.1	51.7 ± 1.8	9.8 ± 1.7	0.62 ± 0.06	71.3 ± 3.1
Breast (n = 7)	42.3 ± 3.3	47.3 ± 2.2[b]	10.2 ± 1.5	0.74 ± 0.10	67.7 ± 3.4
Clavicular (n = 7)	41.1 ± 2.3	49.1 ± 1.9[b]	9.5 ± 1.4	0.70 ± 0.08	68.1 ± 2.3
Abdominal wall (n = 7)	40.2 ± 2.6	49.1 ± 2.4	9.8 ± 2.0	0.68 ± 0.12	68.7 ± 5.0
Anterior thigh (n = 7)	38.9 ± 3.3	50.0 ± 2.2	10.0 ± 1.5	0.65 ± 0.08	70.0 ± 3.8
Medial thigh (n = 7)	38.6 ± 3.3	51.3 ± 2.4	9.5 ± 1.8	0.63 ± 0.10	70.4 ± 4.6
Posterior arm (n = 7)	39.8 ± 3.0	50.0 ± 2.0	9.8 ± 1.5	0.67 ± 0.09	69.6 ± 3.7
Anterior arm (n = 7)	40.9 ± 2.7	49.7 ± 2.2	9.5 ± 1.7	0.69 ± 0.06	68.7 ± 3.6
Intermuscular					
Popliteal (n = 7)	39.8 ± 2.4	50.0 ± 1.9	9.9 ± 1.5	0.66 ± 0.08	69.8 ± 3.3
Trapezius (n = 7)	44.4 ± 4.0	45.4 ± 2.7[b]	9.9 ± 1.7	0.80 ± 0.13	65.2 ± 4.3
Calf (n = 6)	34.1 ± 3.1	55.8 ± 2.2[cdefg]	9.5 ± 1.8	0.52 ± 0.07	74.7 ± 3.7
Intraabdominal and intrathoracic					
Perirenal (n = 7)	43.4 ± 3.4	46.7 ± 1.9[b]	9.4 ± 1.7	0.77 ± 0.11	65.5 ± 4.0
Mesenteric (n = 7)	40.6 ± 1.6	48.3 ± 1.5[b]	9.8 ± 1.1	0.69 ± 0.04	67.9 ± 2.1
Omental (n = 7)	37.8 ± 3.2	51.5 ± 3.6	9.7 ± 1.3	0.62 ± 0.10	70.9 ± 3.6
Pericardial (n = 5)	43.9 ± 4.8	48.7 ± 3.4	7.1 ± 0.9	0.79 ± 0.17	62.9 ± 4.7

[a]Data are mean ± SE from the indicated number of subjects. The index of unsaturation was calculated as the sum of (% unsaturated fatty acid × number of double bonds). Statistical significance ($P < 0.05$) is indicated by [b]*vs.* calf, [c]*vs.* trapezius, [d]*vs.* perirenal, [e]*vs.* mesenteric, [f]*vs.* breast, [g]*vs.* clavicular.

depot contained significantly more monounsaturated fatty acid than the trapezius, perirenal, mesenteric, breast and clavicular adipose depots (Table 4).

DISCUSSION

This study reports the fatty acid composition of triacylglycerols from fifteen distinct adipose depots taken from seven adult male human subjects. The fatty acid composition of a number of these sites has not been reported previously and, to our knowledge, this study represents the largest survey of possible site-specific differences in the fatty acid composition of human adipose tissue yet performed. In accord with previous studies (9–15,30), the major fatty acids found in all adipose depots were oleic, palmitic, linoleic, stearic, myristic and palmitoleic acids; these accounted for approximately 90% of the fatty acids present in each depot. Oleic acid was the most abundant fatty acid, comprising 35 to 45% of the fatty acid in each depot. This is less than the generally accepted proportion of oleic acid in adipose tissue, which is usually reported to be between 45 and 55% (9,12–15,30). However, in this study an isomer of oleic acid, vaccenic acid, accounted for a further 4–5% of fatty acid in each depot (Tables 2). It is likely that in earlier reports oleic and vaccenic acids were included under the single description of "18:1." Indeed, where oleic acid has been specifically referred to separately, its contribution to the adipose fatty acid content is 40–45%, while isomers of oleic acid contributed a further 6–8% (11). These proportions are in agreement with the current study. The proportions of the other major fatty acids reported here are in general agreement with those reported previously (9,12–15,30). In addition to the seven major fatty acids, a number of other fatty acids were identified and quantified. Neither α- nor γ-linolenic acids were detected in the current study, although previous studies have reported that human adipose tissue contains linolenic acid (9,15,30). The content of linolenic acid isomers reported by these earlier studies covers a rather wide range: Malcom *et al.* (15) report that linolenic acid comprises 0.6% of the fatty acid, although it is not clearly identified as n-3 or n-6 or both; Wood *et al.* (30) report that α-linolenic comprises 3% and γ-linolenic 0.2% of fatty acids, although it is stated that neither GC peak was pure linolenic acid; while Field *et al.* (9) give a value of 0.6% for α-linolenic and no value for γ-linolenic.

As has been reported previously (see ref. 31), there was great variation in adipose tissue fatty acid composition between the different subjects (Table 1). The proportions of palmitoleic and linoleic acids differed over a three-fold range between subjects, while the proportions of myristic, palmitoleic, vaccenic and stearic acids differed two-fold between subjects. Adipose tissue fatty acid composition is affected by diet (31), and it is likely that the differences reported here are the result of dietary variation between the subjects; we have no dietary information on any of the subects used in this study.

Site-specific differences in the proportions of palmitoleic, oleic and stearic acids were found (Tables 2 and 3), but the proportions of myristic, palmitic, linoleic and vaccenic acids did not vary between sites (Tables 2). There were no differences in the fatty acid composition of the intraabdominal adipose depots, while the only significant difference between the superficial sites was in the

proportion of stearic acid in the buttock and clavicular depots. The compositions of two intermuscular depots (calf and trapezius) differed greatly from one another. There were a number of differences in the proportions of palmitoleic, oleic and stearic acids between intermuscular (calf) and intraabdominal (perirenal, mesenteric), intermuscular (calf) and superficial (breast, clavicular) and intraabdominal (perirenal, mesenteric) and superficial (buttock, anterior thigh) depots (Tables 2). These differences indicate that some adipose depots, especially calf, trapezius, perirenal and mesenteric, have particular site-specific properties.

Because the rate of adipose tissue triacylglycerol-fatty acid turnover is low, the fatty acid composition of adipose tissue is believed to reflect the dietary fat intake of an individual over the previous twelve months (31). The fact that we (Tables 2 and 3) and others (10-15) find site-specific differences in adipose tissue fatty acid composition indicates that either not all adipose depots turn over their triacylglycerols at the same rate, or that factors other than diet can influence the fatty acid composition of some depots. It is possible that the sites which have been identified as having different fatty acid compositions have specific functions that may dictate the composition of the depot fat. Alternatively, differences in metabolic activity between adipose depots may cause variation in the fatty acid composition. Some site-specific metabolic differences between human adipose depots have been documented (1-8), although only a limited number of anatomical sites have been compared in most studies. A number of metabolic differences between the homologous depots in other mammals have been described (17-25). Interestingly, it has been found that different bovine adipose depots selectively incorporate different fatty acids (24), which would result in different fatty acid compositions. Furthermore, there is a difference in the fatty acid composition between inner and outer regions of the superficial fat depots of both the polar bear (23) and reindeer (25), suggesting that such differences occur within depots, as well as between depots.

Some site-specific differences in human adipose tissue fatty acid composition have been reported previously (10-15), although these studies were often based on a limited number of subjects and anatomical sites (see Introduction). Some studies reported site-specific differences in the proportion of stearic acid only (10) or in the proportions of palmitoleic and stearic acids only (11,12). The current study confirms some of these previous findings, for example that the perirenal depot contains more stearic acid than the buttock depot (10). In addition to differences in palmitoleic and stearic acids, more recent studies have reported some differences in the proportions of myristic, palmitic and oleic acids (13-15). This study did not find any site-specific differences in the proportions of myristic or palmitic acids (Table 2), although there were some differences in the proportions of oleic acid (Tables 2 and 3).

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Inhibition of Phenytoin Bioactivation and Teratogenicity by Dietary n-3 Fatty Acids in Mice[1]

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Evidence suggests that the teratogenicity of the anticonvulsant drug phenytoin (DPH) can result from its bioactivation *via* embryonic prostaglandin synthase and/or maternal cytochromes P450. This study examined whether DPH bioactivation and teratogenicity could be reduced by dietary n-3 fatty acids. Female CD-1 mice were fed diets containing 2 wt% safflower oil and 10 wt% of either hydrogenated coconut oil, safflower oil, or a cod liver oil/linseed oil mixture (CLO/LO) for three weeks prior to impregnation and throughout gestation. DPH (55 or 65 mg/kg) was administered *via* intraperitoneal injections to pregnant mice at 0900 on gestational days 12 and 13, and on day 19 fetuses were given teratologic assessments. A similar dietary study evaluated *in vivo* covalent binding of radiolabeled DPH administered on day 12, and dams were killed 24 h later. A reduction in DPH-induced cleft palates and a decrease in DPH covalent binding to embryonic protein was observed in the CLO/LO group. Feeding CLO/LO enhanced incorporation of n-3 fatty acids into embryos and inhibited embryonic prostaglandin synthase activity. No differences in maternal hepatic cytochromes P450 activities were observed among dietary treatments. These data indicate that dietary n-3 fatty acids could reduce DPH teratogenicity *via* inhibition of embryonic prostaglandin synthase bioactivation of DPH.
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Phenytoin (5,5-diphenylhydantoin, Dilantin, DPH) is one of the most efficacious and widely used anticonvulsants in North America (1). DPH is also a known teratogen in humans (2–4), mice (5,6) and rats (7). In humans, DPH-associated malformations are collectively known as the fetal hydantoin syndrome and characterized by a number of dysmorphic features including cleft lip and palate, malformation of heart and urogenital systems, central nervous system dysfunction and a distinct craniofacial pattern (2,3). Between 10% and 40% of offspring of mothers using the drug during pregnancy have been indicated to have some features of the fetal hydantoin syndrome (2,3).

Although the relationship between DPH metabolism and its teratogenicity remains controversial, there is considerable evidence supporting the involvement of a reactive intermediate in the teratogenicity of DPH. DPH has been shown to bind covalently to embryonic, placental and hepatic tissues indicating bioactivation to a reactive intermediate has occurred (6,8), and significantly higher levels of fetal DPH covalent binding has been observed in fetuses with cleft palate (6). *In vivo* and *in vitro* evidence from a number of human and animal studies have suggested that the teratogenic activity of DPH could result from both cytochromes P450-mediated bioactivation of DPH to an arene oxide intermediate (4,6) and prostaglandin synthase-catalyzed bioactivation of DPH to a reactive free radical (9,10). For instance, covalent binding of a reactive intermediate of DPH to proteins has been shown to be catalyzed by NADPH-dependent cytochromes P450 activity in rodent liver microsomes (8,9). In addition, incubations containing purified prostaglandin synthase or murine hepatic microsomes incubated with arachidonic acid have also stimulated *in vitro* covalent binding of DPH to proteins (9). Potentially important enzymatic pathways of detoxification that have been demonstrated in DPH teratogenicity include hydration of the arene oxide by epoxide hydrolase (4,8) and interactions involving glutathione (11). Some paradoxical observations, however, have suggested difficulties with the arene oxide hypothesis (5,10). For example, pretreatment with phenobarbital, an inducer of cytochromes P450, inhibited DPH teratogenicity in pregnant mice, whereas SKF 525A, an inhibitor of cytochromes P450, increased DPH teratogenicity (5). On the other hand, pretreatment of mice with aspirin, an irreversible inhibitor of the cyclooxygenase component of prostaglandin synthase, markedly inhibited the *in vivo* teratogenic effects of DPH (10). DPH-induced embryopathy and covalent binding of DPH have been observed in murine embryo cultures in the absence of maternal bioactivating enzymes indicating a role for embryonic bioactivation of DPH to a teratogenic reactive intermediate (12). Prostaglandin synthase bioactivation of DPH could be an important teratogenic mechanism that takes place at the embryonic level since at early stages of development embryonic prostaglandin synthase activity is high (13), while embryonic cytochromes P450 have been shown to be low or negligible (14).

The potential of dietary n-3 fatty acids to reduce DPH teratogenicity *via* inhibition of embryonic prostaglandin synthase bioactivation of DPH was evaluated in the present study. Although it has been well demonstrated that feeding a diet rich in n-3 fatty acids reduces the prostaglandin synthesizing capacity of a variety of tissues (15–17), little is known concerning the effect of these diets on embryonic prostaglandin synthase activity. The effect of n-3 fatty acid diets on maternal liver cytochromes P450 content and activities was also studied since diet-induced alterations in these enzyme activities could modulate DPH metabolism and teratogenicity.

MATERIALS AND METHODS

Animals, dietary treatments and animal breeding. Virgin female CD-1 mice (Charles River Canada, Inc., St. Constant, Quebec, Canada), with body weights 20–25 g, were

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Abbreviations: CLO/LO, cod liver oil/linseed oil; DPH, phenytoin; FAME, fatty acid methyl esters; HCO, hydrogenated coconut oil; $PGA_{2\alpha}$, prostaglandin $A_{2\alpha}$; $PGE_{2\alpha}$, prostaglandin $E_{2\alpha}$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PUFA, polyunsaturated fatty acids; SAFF, safflower oil.

housed in plastic cages with ground cob bedding (Beta Chips, Northeastern Products Corp., Warrensburg, NY). Animals were kept in a temperature-controlled room with a 12-h light-dark cycle automatically maintained (light cycle 0700 to 1900). After 3 d of feeding a nonpurified diet (Purina Mouse Chow, Ren's Feed and Supply, Oakville, Ontario, Canada), mice were weighed and randomly assigned into one of three dietary regimens of purified diets. Each group of mice was fed a modified AIN-76A diet with the following dry ingredients (g/100 g): casein (vitamin-free), 18.0; methionine, 0.03; glucose, 56.1; α-cellulose, 5.0; AIN-76 mineral mix, 6.0; AIN-76 vitamin mix, 2.2; and choline chloride, 0.4. Added to these dry ingredients was either hydrogenated coconut oil (HCO), safflower oil (SAFF) or a mixture (1:1) of cod liver oil and linseed oil (CLO/LO) such that the diets contained 10% fat by weight. All diets were supplemented with 2 g/100 g safflower oil to provide essential fatty acids in the diets, such that final diets contained 12% fat by weight. In addition, *dl*-α-tocopheryl acetate was added at 7.4 mg/g oil. All dietary components were supplied by ICN Nutritional Biochemicals (Cleveland, OH). The fatty acid profile of the diets is shown in Table 1. Autoxidation of the oils was prevented by the addition of synthetic antioxidant (0.01% butylated hydroxytoluene, Sigma Chemical Co., St. Louis, MO) to the oils. Fresh diet was provided every other day, and uneaten food was discarded. Food intake was recorded every third day throughout the experiment. Mice were fed the diets for at least three weeks prior to mating and were maintained on the diets throughout gestation. Three females were housed with one male mouse of the same strain overnight between 1500 and 0900. Pregnancy was ascertained the next morning by the presence of a vaginal plug, and this time was designated as gestational day 1. Food and water was provided *ad libitum* throughout the experiment. Fresh diets were stored in sealed containers, flushed with nitrogen and stored at 4°C until used. Lipid peroxidation of diets either stored at −20°C or kept at room temperature for 48 h did not increase as measured by the thiobarbituric acid assay (18).

To obtain data on phospholipid fatty acid profiles, and on P450 and prostaglandin synthase activities, mice receiving dietary treatments alone were killed by cervical dislocation on gestational day 12. All embryos were surgically removed from the dams, dissected free from their investing membranes in 9 g/L saline, weighed, immediately frozen in liquid nitrogen and stored at −80°C until assayed. Maternal livers and placenta were also removed immediately, washed in isotonic saline, blotted on paper to remove excess fluid, weighed, frozen in liquid nitrogen and stored at −80°C until analysis.

Drug treatments. DPH was dissolved in normal saline containing 2 mM sodium hydroxide (Fisher Scientific Co., Toronto, Ontario, Canada) at a final pH of 10.5. Teratogenic doses of DPH of either 55 or 65 mg/kg, were administered *via* intraperitoneal injection to 15 to 20 dams in each HCO, SAFF and CLO/LO treatment group as a two-dose regimen at 1100 on gestational days 12 and 13. Saline controls received intraperitoneal injections of sterile physiological saline instead of DPH. Dams were killed for toxicological assessment by cervical dislocation on gestational day 19, one day before spontaneous delivery. In a second study, in order to examine dietary fat effects on covalent binding of DPH *in vivo*, tritiated DPH (5,5-[*phenyl*-4-^{3}H(*N*)] diphenylhydantoin, 51.5 Ci/mmol; New England Nuclear, Lachine, Quebec, Canada) was mixed with unlabeled DPH and administered, 1 μCi/g body weight, on gestational day 12 to four dams in each of the dietary treatment groups. All solutions were prepared immediately prior to administration and were constituted to maintain a standard injection volume of 10 mL/kg body weight.

TABLE 1

Fatty Acid Composition of Dietary Fats

Fatty acid[b]	Diet[a] HCO	SAFF	CLO/LO[c]
14:0	29.3	0.9	3.1
16:0	17.1	7.2	10.7
16:1n-7	trace[d]	n.d.[e]	2.9
18:0	5.1	2.3	3.1
18:1n-9	33.7	11.2	19.8
18:2n-6	14.6	78.4	18.1
20:4n-6	n.d.	n.d.	1.1
18:3n-3	trace	n.d.	30.9
18:4n-3	n.d.	n.d.	1.1
20:5n-3	n.d.	n.d.	4.9
22:5n-3	n.d.	n.d.	trace
22:6n-3	n.d.	trace	4.0
Monounsaturated, %	34	11	23
Saturated, %	51	10	17
Polyunsaturated, %	15	78	60
P/S ratio[f]	0.3	7.5	3.6
Unsaturated index[g]	63	168	211

[a]HCO, hydrogenated coconut oil; SAFF, safflower oil; CLO/LO, cod liver oil/linseed oil.
[b]Fatty acids are identified by the number of carbon atoms and the number of double bonds, followed by the position of the first double bond from the methyl end of the fatty acid.
[c]Mixture of 1 part cod liver oil to 1 part linseed oil.
[d]Trace (<0.5%).
[e]Not detectable.
[f]Polyunsaturated to saturated fatty acid ratio.
[g]Unsaturation index is the sum of percentages of individual fatty acids multiplied by the number of double bonds.

Teratological assessment. Following laparotomy on day 19 of gestation, the uterus was exteriorized, and the number and location of resorptions and each fetus were noted and fetuses were sexed and weighed. After being fixed for at least two days in Carnoy's solution, a transverse incision was made between the upper and lower jaw of each fetus and the palatal shelves were examined with the unaided eye for the presence or absence of a cleft.

Covalent binding studies. Radiolabeled DPH was administered with unlabeled DPH at 1100 on gestational day 12. Dams were killed at 1100 on gestational day 13, and the amount of radiolabeled DPH covalently bound to protein in maternal and embryonic tissues was determined. For the determination of covalent binding of tritiated DPH, all placenta and embryos from each litter were pooled separately whereas livers from all dams were assayed individually. The determination of covalent binding of tritiated DPH involved a validated modification of an exhaustive washing method to permit more accurate determinations to microgram quantities of embryonic tissue (9). Tissue samples from maternal liver, placenta and embryos were homogenized in ice-cold KCl 1.15 g/L for 20 s with a probe homogenizer (Ultra-Turrax, Janke and Kundel GmbH, Staufen, Germany). As previously

described (9), proteins were precipitated, washed and digested for counting of covalently bound radiolabeled DPH. The amount of protein in each sample was assayed by a modified method of Lowry (19), and the level of covalently bound radiolabeled DPH was standardized per mg protein.

Extraction of lipids and tissue fatty acid analysis. For fatty acid analysis, maternal livers, placenta and embryos were thawed and homogenized in chloroform/methanol (2:1, vol/vol) containing 0.005 g/L (wt/vol) butylated hydroxytoluene following the procedures by Folch *et al.* (20). Tissue phospholipids were separated by adsorption chromatography on silica cartridges 25 mm × 10 mm i.d. (Sep-pack, Supelco, Oakville, Ontario, Canada) by the method described by Juaneda and Rocquelin (21). Fatty acid methyl esters (FAME) of total phospholipids were prepared with boron trifluoride methanol reagent according to Morrison and Smith (22). FAME were separated and quantified by gas-liquid chromatography (Varian 3700 gas chromatograph fitted with dual FID detectors—Varian, Palo Alto, CA) with 1.82 m × 6.3 mm packed glass column packed with 10% Silar 10 CP on 100/120 chromosorb Supelcoport (Supelco, Oakville, Ontario, Canada) and a Shimadzu C-R6A Chromatopac integrator (Kyoto, Japan). Identification of the fatty acid methyl esters was by comparison of retention times with those of authentic standards (Nu-Chek Prep, Elysian, MN).

Prostaglandin $PGF_{2\alpha}$ determinations. Production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by embryonic homogenates was determined according to a modification of the method of Klein *et al.* (23). Frozen embryos were placed into a polypropylene test tube and quickly homogenized in the semi-frozen state by vortexing for 20 s. Homogenates were incubated at 37°C for 1 h in a shaking water bath. Following incubation, 4 mL of absolute ethanol was placed into each tube and incubated in an ice bath for 30 min. The samples were centrifuged for 20 min at 1600 × *g* and the supernatant was collected. The supernatant was dried under nitrogen and reconstituted in absolute ethanol. The pellets were saved to determine their protein content (19) and to measure the DNA content using a fluorometric assay (24). Levels of $PGF_{2\alpha}$ of the supernatant were determined by a double antibody (Method 2) radioimmunoassay kit using rabbit antiprostaglandin $PGF_{2\alpha}$ serum (Incstar Corporation, Stillwater, MN) according to the manufacturer's instructions. This antiserum is reported by the manufacturer to have minimal cross-reactivity (less than 2%) with prostaglandin $A_{2\alpha}$ ($PGA_{2\alpha}$), prostaglandin $E_{2\alpha}$ ($PGE_{2\alpha}$) and $PGF_{2\alpha}$ metabolite. The levels of $PGF_{2\alpha}$ assayed are expressed as ng of $PGF_{2\alpha}$ synthesized per gram of embryonic protein.

Cytochromes P450 content and activities. After dams were killed, their livers were removed and stored at −80°C until analysis. Liver microsomes were prepared by differential centrifugation as previously described (9). Concentration of cytochromes P450 was determined in microsomes diluted to 2.0 g protein/L with 0.1 mol/L phosphate buffer using the Beckman DU-40 spectrophotometer (Beckman, Fullerton, CA) according to the method of Omura and Sato (25). P450 isozyme activities were assayed using a Sequoia-Turner (Mountain View, CA) single beam fluorescence spectrophotometer, with the excitation wavelength set at 530 nm, the emission wavelength set at 585 nm, and the entrance and exit slits set at 10 nm. 7-Pentoxyresorufin *O*-dealkylase activity was assayed at 11.2 μmol/L 7-pentoxyresorufin (final concentration) according to Lubet *et al.* (26). The activity of 7-ethoxyresorufin *O*-deethylase was determined using a final concentration of 0.9 μmol/L 7-ethoxyresorufin (27). Linear increases were measured for several minutes and compared to resorufin used as a standard.

Statistical analyses. Statistical analyses were performed on both an individual fetus and a litter basis. The incidence of cleft palates was calculated as the number of fetuses with cleft palates divided by the number of fetuses and the resorption incidence as the number of resorptions divided by the number of implantation sites (fetuses plus resorptions). Incidence data (*i.e.*, cleft palates and resorptions) were analyzed by Chi-square. Statistical comparisons of differences between groups were analyzed by one-way ANOVA using the Statistical Analysis System for personal computers (SAS Institute, Cary, NC). As fatty acid and covalent binding determinations were conducted on the pooled embryos and placenta from a litter, the litter represents the unit of analysis for these measures. The embryo was used as the unit of analysis for prostaglandins, protein and DNA content. When significant differences occurred, the treatment mean differences were identified by Tukey's test. A probability of $P < 0.05$ was accepted as the minimal level of significance.

RESULTS

Teratology. Figure 1 shows the teratogenic responses to doses of DPH (55 and 65 mg/kg/day) given on days 12 and 13 of gestation to mice fed the SAFF, HCO, and CLO/LO diets. Mice given the SAFF and HCO diets had approximately 19% and 21% incidences of cleft palate after the 55 mg/kg DPH dose treatment. Mice fed the CLO/LO diet, however, showed approximately a 72% lowered incidence of cleft palate as compared to the other dietary groups. Fetal weight and the resorption frequency were not affected by the source of dietary fats in DPH-treated mice (Fig. 1). After the 65 mg/kg dose treatment of DPH, mice fed the SAFF and HCO diets had cleft palate incidences of 42% and 41%, respectively. In contrast, the response of cleft palates to the higher dose of DPH was inhibited in mice fed the CLO/LO diet which showed an incidence of 28% cleft palate (Fig. 1). The resorption frequency was not affected by any of the dietary treatments; however, fetal weight showed a slight decrease (6%) in the DPH-treated dams receiving the CLO/LO diet (Fig. 1). The vehicular solution for DPH which was composed of NaOH (2 mM/L) in normal saline did not cause cleft palates in any of the dietary treatment groups, and the saline-treated mice showed a low incidence of resorptions (4.2%). The mean fetal weight of the saline controls was 1.29 ± 0.01 g (mean ± SEM).

Covalent binding. The covalent binding of radiolabeled DPH to maternal and embryonic tissues measured on gestational day 13 is shown in Figure 2. The CLO/LO dietary treatment resulted in a 33% decrease in covalent binding of radiolabeled DPH to embryonic protein ($P < 0.05$) relative to the other dietary treatments, with no effect on binding to the associated placentas. Conversely, the CLO/LO diet increased covalent binding of DPH in maternal liver relative to the SAFF dietary group ($P < 0.05$).

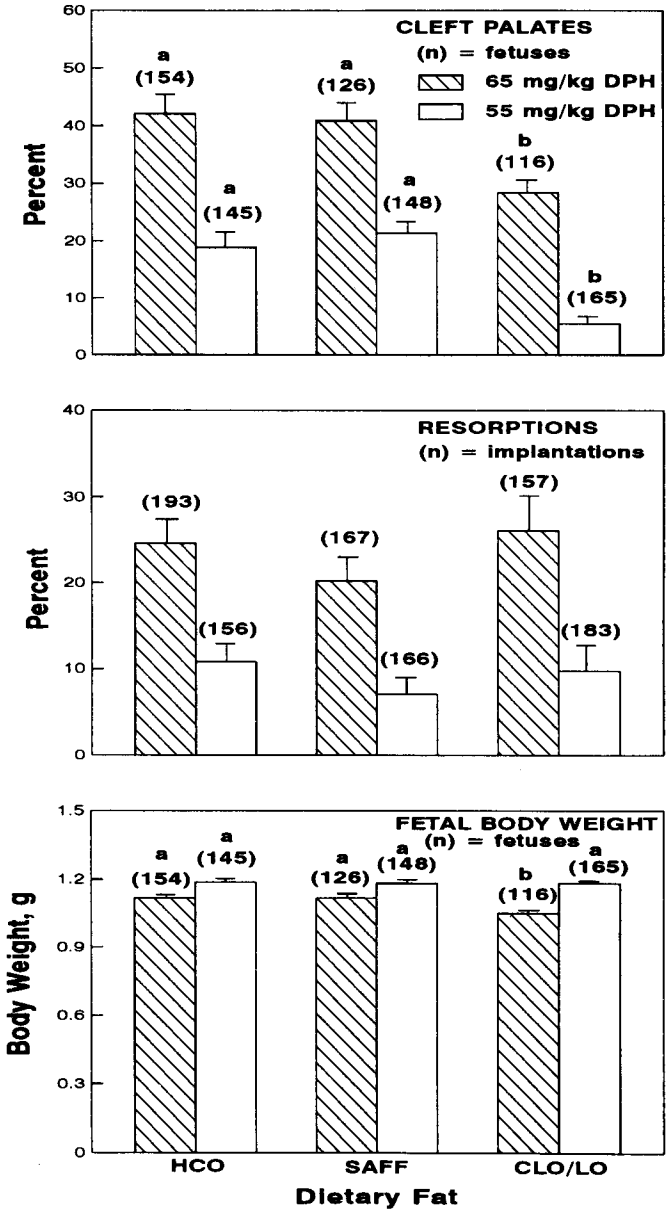

FIG. 1. The effect of modifications in dietary fats on DPH embryopathy. Groups of 15 to 20 CD-1 mice were fed diets containing 2 wt% safflower oil (SAFF) and 10 wt% of either hydrogenated coconut oil (HCO), SAFF, or a cod liver oil/linseed oil mixture (CLO/LO) for three weeks prior to impregnation and throughout gestation. At 0900 on gestational days 12 and 13 mice were administered 55 or 65 mg/kg DPH i.p. Each bar represents the mean plus the standard error for the number of embryos given in parentheses. Values for a dietary treatment with a particular drug dose with different letters are significantly different ($P < 0.05$) as determined by ANOVA with means separated by Tukey's test.

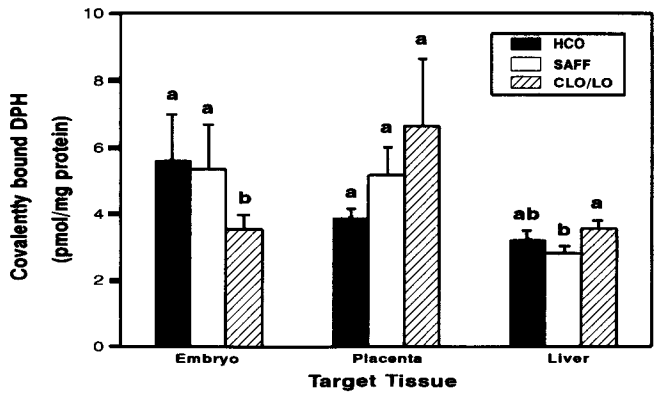

FIG. 2. Effect of modifications in dietary fats on the covalent binding of DPH to embryonic, placental and maternal liver tissues. Groups of five CD-1 mice were fed diets containing 2 wt% safflower oil (SAFF) and 10 wt% of either hydrogenated coconut oil (HCO), SAFF, or a cod liver oil/linseed oil mixture (CLO/LO) for three weeks prior to impregnation and throughout gestation. Pregnant mice were administered DPH, 65 mg/kg i.p., at 1100 on gestational day 12. The dose of radiolabeled DPH was 1 μCi/g body weight. Dams were killed 24 h later. Each bar represents the mean plus the standard error; n = 4. Values for a particular dietary treatment with different letters are significantly different ($P < 0.05$) as determined by ANOVA with means separated by Tukey's test.

Effects of diet on gestational indices, food intake, protein and DNA content. All diets were well accepted by the female mice, and no differences were observed in terms of food intake between the dietary treatments (6 g/d per mouse). Maternal consumption of a diet containing CLO/LO resulted in gestational day 12 embryo weights (0.089 ± 0.003 g) (mean ± SEM) that were significantly ($P < 0.05$) lower than those of mouse pups fed a diet containing either SAFF (0.100 ± 0.003 g) or HCO (0.096 ± 0.005 g) as fat sources. The mean DNA content per embryo was significantly lower in the CLO/LO embryos on gestational day 12, although the ratio of protein to DNA was increased in CLO/LO embryos relative to the other dietary treatments (Table 2). The growth retardation was not likely the consequence of lower maternal weight gain as no significant differences were also observed between the HCO and CLO/LO diet groups in terms of mean body weight of the dams on gestational day 12 (data not shown). The total number of implantation sites per dam, the number of embryos per litter, placental weights and resorptions per litter did not differ between dietary treatments (data not shown).

Fatty acid composition of total phospholipids in maternal livers, placenta and day 12 embryos. The polyunsaturated fatty acid (PUFA) composition of the maternal hepatic, placental and embryonic phospholipids was highly dependent upon the composition of dietary lipid (Table 3). A comparison of the three dietary groups reveals that CLO/LO resulted in a significant increase in embryonic, placental and maternal liver phospholipid content of C_{20}n-3 and C_{22}n-3 fatty acids. The concentrations of 20:5n-3, 22:5n-3 were detectable only in embryos of CLO/LO-fed animals, and embryonic concentrations of 22:6n-3 increased approximately four-fold in comparison to the HCO and SAFF treatments. The incorporation of n-3 fatty acids in all the tissues was associated with a decrease in 20:4n-6 relative to that found in the HCO- and SAFF-fed groups (Table 3). The concentration of 20:4n-6 was similar in both HCO- and SAFF-fed groups. Levels of 18:2n-6 in liver phospholipids reflected dietary availability of this fatty acid, with SAFF mice accumulating greater amounts relative to the other dietary groups. The levels of 18:2n-6, however, did not vary in embryonic and placental phospholipids between SAFF and CLO/LO dietary treatments although the dietary HCO group showed depressed levels as compared to the other dietary

TABLE 2

DNA and Protein Contents of Day 12 Embryos from Mice Fed Three Different Sources of Fat

	Dietary fat		
	HCO[a]	SAFF	CLO/LO
Total DNA, μg	30.28 ± 1.33[b]	30.83 ± 0.98[b]	22.55 ± 2.90[c]
Total protein, mg	2.84 ± 0.26[b]	3.56 ± 0.14[b,c]	3.81 ± 0.23[c]
Total protein/total DNA	4.87 ± 0.39[b]	5.80 ± 0.24[c]	7.97 ± 0.88[d]

[a]Pregnant mice were fed diets containing 2 wt% safflower oil (SAFF) and 10 wt% of hydrogenated coconut oil (HCO), SAFF, or a cod liver oil/linseed oil (CLO/LO) mixture for three weeks prior to impregnation and until gestational day 12.

[b–d]Mean ± SEM, n = 10; different letters denote statistical significance (ANOVA; $P < 0.05$). When significant differences occurred ($P < 0.05$), treatment mean differences were identified by Tukey's test.

groups (Table 3). The feeding of the CLO/LO and SAFF diets was also accompanied by lowered levels of 16:1n-7 and 18:1n-9 when compared with animals fed diets containing HCO. The phospholipid content of embryos, placenta and livers of the CLO/LO group was found to have markedly higher n-3 concentrations and n-3/n-6 ratios and lower n-6 concentrations as compared to the other diet groups. Dietary treatment had no effect on the ratio of PUFA to saturated fatty acids or the unsaturation index in embryo phospholipids, despite large differences in these indices in the diet. Conversely, the CLO/LO diet induced significant increases in the PUFA content, polyunsaturated to saturated ratio and unsaturation index in membrane phospholipids of placental and hepatic tissues (Table 3).

Prostaglandin measurements. Embryo tissue synthesis of prostaglandin $F_{2\alpha}$ from the three diet groups are depicted in Figure 3. Consistent with the lower levels of 20:4n-6 and higher concentrations of 20:5n-3 in the total phospholipids of CLO/LO embryos, CLO/LO embryo homogenates synthesized markedly lower levels of prostaglandin $F_{2\alpha}$ than did embryo homogenates from the other diet groups after 1 h of incubation at 37°C. No significant differences in prostaglandin $F_{2\alpha}$ levels were observed between SAFF and HCO embryos.

Effects of diet on microsomal enzymes. The effects of dietary treatments on cytochromes P450 content and activities in maternal livers are shown in Table 4. There was no significant dietary effect on cytochromes P450 content. The constitutive levels of the activities of microsomal *O*-dealkylation of 7-pentoxyresorufin and *O*-deethylation of 7-ethoxyresorufin were low, and no significant differences were observed among dietary groups (Table 4).

DISCUSSION

The inhibition of DPH-induced cleft palate together with the decreased covalent binding of radiolabeled DPH to embryonic protein in mice fed the CLO/LO diet suggests that the teratologic inhibition was due to decreased embryonic exposure to the reactive intermediate of DPH. The decrease in cleft palate incidence in the CLO/LO group occurred without any change in the incidence of resorptions. This is noteworthy since detection of cleft palates would have been precluded by a high resorption incidence in the CLO/LO group which could have led to artifactually lowered estimates of teratogenicity.

The present study demonstrated that tissue phospholipids of embryos, placenta and liver reflected high maternal intakes of n-3 fatty acids following consumption of the CLO/LO mixture for approximately five weeks. This was anticipated since previous studies have demonstrated significant increases in 20:5n-3 and 22:6n-3 content and a reduction in 20:4n-6 in lung phospholipids of newborn offspring of rats fed high n-3 PUFA diets for three weeks (16,17). The relationship of a high n-3 fatty acid content in membrane phospholipids of day 12 murine embryos with decreased embryonic prostaglandin synthase activity is a new observation although prostaglandin synthesis has been detected in day 11 rat embryo homogenates (23) and reduced prostaglandin synthase activity has been demonstrated in lung tissue of newborns of n-3 fatty acid-fed dams (17).

The reduction of embryonic prostaglandin synthase activity in the CLO/LO diet group provides support for the concept that a mechanism by which CLO/LO embryos were protected against DPH teratogenicity involved inhibition of embryonic prostaglandin synthase bioactivation of DPH. Increased tissue n-3 fatty acid content can inhibit prostaglandin synthase activity *via* several mechanisms including: (i) reduced levels of 20:4n-6 availability (28); (ii) an elevation of n-3 fatty acids, particularly eicosapentaenoic acid (20:5n-3), which directly competes with 20:4n-6 as a substrate for cyclooxygenase (28,29); and (iii) a direct inhibition in cyclooxygenase activity by n-3 fatty acids *via* decreased peroxide tone (30). The comparable prostaglandin synthesizing activities observed between HCO and SAFF embryos are consistent with the equivalent concentrations of the 20:4n-6 precursor present in the embryonic membrane phospholipids. The mice fed the HCO and SAFF diets elongated and desaturated 18:2n-6 to a similar degree as reflected by the equivalent amounts of 20:4n-6 in embryonic, placental and liver tissue phospholipids of HCO- and SAFF-fed mice. These findings support and extend the idea that desaturation and chain elongation of 18:2n-6 is regulated such that increasing dietary intake of 18:2n-6 above a threshold value of approximately between 1 and 2% of energy does not lead to any further increase in 20:4n-6 levels (31). Similar findings have been reported by other investigators who demonstrated unchanged prostaglandin synthesis in renal homogenates despite twenty-fold increases in dietary linoleic acid above 0.4 wt% (31).

The present dietary studies support previous work by

TABLE 3

Fatty Acid Composition of Embryo, Placenta and Liver Total Phospholipids Isolated from Mice Fed Diets Containing 10 wt% of Either Hydrogenated Coconut Oil (HCO), Safflower Oil (SAFF), or a Cod Liver Oil/Linseed Oil Mixture (CLO/LO)[a]

Fatty acid	Tissue	Dietary fat		
		HCO	SAFF	CLO/LO
		g/100 g fatty acids		
14:0	Embryo	1.46 ± 0.10	1.83 ± 0.06	2.16 ± 0.68
	Placenta	0.81 ± 0.21	0.47 ± 0.08	0.24 ± 0.10
	Liver	0.23 ± 0.05[b]	0.07 ± 0.03[c]	0.01 ± 0.01[c]
16:0	Embryo	37.11 ± 5.55	35.64 ± 0.26	36.88 ± 4.34
	Placenta	21.86 ± 2.49	23.54 ± 0.48	23.26 ± 0.85
	Liver	26.40 ± 0.63	24.55 ± 0.50	25.41 ± 2.34
16:1n-7	Embryo	4.45 ± 0.30	4.11 ± 0.21	4.08 ± 0.35
	Placenta	1.54 ± 0.58	2.41 ± 1.92	0.66 ± 0.85
	Liver	0.90 ± 0.08[b]	0.21 ± 0.04[c]	0.35 ± 0.06[c]
18:0	Embryo	15.46 ± 1.75	17.21 ± 0.29	15.35 ± 1.43
	Placenta	26.32 ± 1.73	28.56 ± 1.05	25.69 ± 1.28
	Liver	27.02 ± 0.49	28.82 ± 0.55	26.13 ± 2.78
18:1n-9	Embryo	21.89 ± 1.11	19.6 ± 0.55	21.55 ± 0.68
	Placenta	12.20 ± 1.14[b]	8.71 ± 0.30[c]	10.76 ± 0.78[b,c]
	Liver	7.33 ± 0.50[b]	4.16 ± 0.58[c]	4.04 ± 0.48[c]
18:2n-6	Embryo	2.39 ± 0.24[b]	4.49 ± 0.15[c]	3.79 ± 0.20[c]
	Placenta	7.64 ± 0.82[b]	13.61 ± 0.54[c]	12.23 ± 0.78[c]
	Liver	8.89 ± 0.64[b]	15.28 ± 0.82[c]	11.83 ± 1.23[b]
20:2n-6	Embryo	1.12 ± 1.12	0.76 ± 0.76	n.d.
	Placenta	0.47 ± 0.20	0.91 ± 0.24	0.21 ± 0.13
	Liver	0.11 ± 0.06	0.25 ± 0.21	n.d.
20:3n-6	Embryo	n.d.	n.d.	n.d.
	Placenta	0.58 ± 0.24	0.69 ± 0.20	0.78 ± 0.20
	Liver	0.30 ± 0.06[b]	0.26 ± 0.08[b]	0.59 ± 0.08[c]
20:4n-6	Embryo	15.88 ± 3.34	15.38 ± 0.38	8.11 ± 1.82
	Placenta	20.62 ± 1.92[b]	18.39 ± 1.10[b]	7.30 ± 0.42[c]
	Liver	23.41 ± 1.48[b]	22.48 ± 0.78[b]	9.82 ± 1.89[c]
18:3n-3	Embryo	n.d.	n.d.	n.d.
	Placenta	n.d.	0.07 ± 0.07	2.13 ± 2.13
	Liver	0.15 ± 0.07[b]	0.63 ± 0.19[c]	0.33 ± 0.10[b,c]
20:5n-3	Embryo	n.d.[b]	n.d.[b]	0.74 ± 0.30[c]
	Placenta	n.d.[b]	0.50 ± 0.50[b]	4.52 ± 0.27[c]
	Liver	0.60 ± 0.60[b]	n.d.[b]	5.53 ± 1.00[c]
22:5n-3	Embryo	n.d.	n.d.	1.48 ± 0.72
	Placenta	0.30 ± 0.30[b]	1.16 ± 1.16[b]	4.95 ± 0.67[c]
	Liver	0.09 ± 0.09[b]	n.d.[b]	0.69 ± 0.07[c]
22:6n-3	Embryo	1.15 ± 0.55[b]	1.46 ± 0.10[b]	6.57 ± 1.65[c]
	Placenta	1.98 ± 0.41[b]	2.53 ± 0.33[b]	10.84 ± 0.93[c]
	Liver	0.75 ± 0.75[b]	n.d.[b]	5.58 ± 0.91[c]
n-3	Embryo	1.14 ± 0.55[b]	1.46 ± 0.10[b]	8.80 ± 2.33[c]
	Placenta	1.18 ± 0.69[b]	4.26 ± 1.94[b]	22.45 ± 2.55[c]
	Liver	1.60 ± 1.43[b]	0.63 ± 0.19[b]	12.13 ± 1.74[c]
n-6	Embryo	1.39 ± 3.33[b,c]	20.63 ± 0.66[b]	11.90 ± 1.99[c]
	Placenta	29.31 ± 2.83[b]	33.60 ± 1.03[b]	20.52 ± 0.56[c]
	Liver	32.70 ± 1.43[b]	38.27 ± 0.64[b]	22.25 ± 2.51[c]
n-3/n-6	Embryo	0.05 ± 0.02[b]	0.07 ± 0.01[b]	0.68 ± 0.20[c]
	Placenta	0.07 ± 0.02[b]	0.12 ± 0.05[b]	1.09 ± 0.14[c]
	Liver	0.08 ± 0.08[b]	0.02 ± 0.01[b]	0.61 ± 0.08[c]
ΣSatur.	Embryo	54.76 ± 4.45	54.68 ± 0.53	54.39 ± 3.38
	Placenta	49.00 ± 4.21	52.57 ± 1.52	49.20 ± 2.00
	Liver	53.64 ± 0.84	53.44 ± 0.89	51.56 ± 5.07
ΣMonounsat.	Embryo	26.34 ± 0.99	23.72 ± 0.53	25.63 ± 0084
	Placenta	13.73 ± 1.35	11.11 ± 1.86	12.56 ± 1.01
	Liver	8.22 ± 0.56[b]	4.37 ± 0.60[c]	4.52 ± 0.50[c]
ΣPolyunsat.	Embryo	20.54 ± 3.74	22.10 ± 0.62	20.70 ± 3.88
	Placenta	31.59 ± 3.31[b]	37.85 ± 2.70[b,c]	42.97 ± 2.54[c]
	Liver	38.21 ± 1.24[b]	42.46 ± 0.56[b,c]	47.78 ± 2.87[c]
P/S ratio[b]	Embryo	0.41 ± 0.10	0.40 ± 0.01	0.40 ± 0.08
	Placenta	0.64 ± 0.04[b]	0.72 ± 0.06[b,c]	0.88 ± 0.07[c]
	Liver	0.72 ± 0.04[b]	0.79 ± 0.02[b]	0.95 ± 0.04[c]
Unsat. index[c]	Embryo	104 ± 16	105 ± 1	116 ± 18
	Placenta	127 ± 13[b]	139 ± 11[b]	187 ± 11[c]
	Liver	157 ± 8[b]	149 ± 3[b]	215 ± 11[c]

[a]Pregnant mice were fed diets containing 2 wt% safflower oil (SAFF) and 10 wt% of either hydrogenated coconut oil (HCO), SAFF, or a cod liver oil/linseed oil mixture (CLO/LO) for three weeks prior to impregnation and until day 12 of gestation. The values are expressed as means ± SEM of five litters of pooled embryos or pooled placenta and nine maternal livers in each group; values in a row for a particular tissue with different superscript letters (b,c) are significantly different ($P < 0.05$) as determined by ANOVA and Tukey's test comparisons; n.d. = not detectable.

[b]Polyunsaturated to saturated fatty acid ratio.

[c]Unsaturation index is the sum of percentages of individual fatty acids multiplied by the number of double bonds.

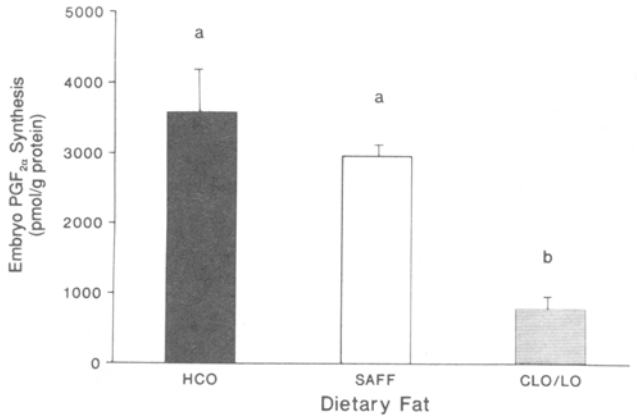

FIG. 3. $PGF_{2\alpha}$ production by homogenates of day 12 embryos removed from mice fed three different sources of fat. Pregnant mice were fed diets containing 2 wt% safflower oil (SAFF) and 10 wt% of either hydrogenated coconut oil (HCO), SAFF, or a cod liver oil/linseed oil mixture (CLO/LO) for three weeks prior to impregnation and until day 12 of gestation. Values are expressed as means ± SEM; n = 10. Values for a particular dietary fat with different letters are significantly different ($P < 0.05$) as determined by ANOVA with means separated by Tukey's test comparisons.

Wells *et al.* (10) showing a marked reduction in DPH-induced cleft palates and covalent binding in CD-1 mice following pretreatment by acetylsalicylic acid, a potent inhibitor of prostaglandin synthase activity. Correspondingly, acetylsalicylic acid pretreatment did not protect against DPH-induced resorptions and reductions in fetal body weight, further suggesting that a similar protective mechanism was involved in both studies. As prostaglandin synthase activity is high in embryonic (13) and certain extrahepatic tissues (9), this pathway may contribute substantially to DPH bioactivation in these tissues.

The lack of protective effects of the CLO/LO diet against DPH-induced fetal resorptions and weight loss suggests that other mechanisms could be involved in these toxicities. In fact, a small (6%) but statistically significant decrease in mean fetal weight was observed in the CLO/LO dietary group following administration of 65 mg/kg DPH. This result may be biologically significant since a decreased embryo weight was observed in the untreated CLO/LO dietary group on gestational day 12. Embryonic DNA concentrations on gestational day 12 were also lowered in the CLO/LO embryos indicating that cell proliferation was delayed and that gestational delay in organ development in CLO/LO embryos may have taken place. This finding suggests the possibility that delayed organ growth of CLO/LO embryos may have been in part responsible for the decreased cleft palate incidence by delaying the critical period of susceptibility to DPH-induced cleft palate. Fetal growth retardation has been previously observed following feeding n-6 fatty acid deficient HCO diets or diets with a high ratio of n-3 to n-6 fatty acids (16). The cause of growth retardation in developing embryos which are accompanied by high n-3 to n-6 fatty acid ratios is unclear. One possible mechanism by which n-3 rich diets may exert growth-suppressing effects may be mediated *via* a depression in the growth stimulating action of prostanoids since PGE_2 has been found to stimulate DNA synthesis and growth of cultured cells (32).

The present observations contradict a recent proposal (33) that DPH disrupts craniofacial development by inhibiting arachidonic acid release from membrane phospholipids for cyclooxygenase-mediated conversion into prostaglandins. This hypothesis was tested by Kay *et al.* (33) by incubation of exogenous arachidonic acid in embryo cultures treated with DPH. Although a marked reduction in gross morphological defects was noted in cultures containing arachidonic acid, the capability of exogenous arachidonic acid to stimulate cyclooxygenase activity in the embryos was not assessed. In contrast, the present study indicates that a decreased incorporation of arachidonic acid into embryonic tissue phospholipids and a reduction in embryonic prostaglandin synthesizing capacity was associated with an abatement of DPH-induced cleft palate. Moreover, recent embryo culture experiments from our laboratory have indicated that mouse embryos from CLO/LO dams demonstrated decreased morphological defects following incubation with DPH in comparison with SAFF embryos which were treated with DPH at the same embryological age (High, K. and Kubow, S., unpublished data).

The content and activities of liver microsomal cytochromes P450 were investigated since the activities of this system are known to be influenced by the fatty acid composition of phospholipids of microsomal membranes (34,35) and as altered cytochromes P450 activities could alter DPH metabolism and teratogenicity. As CLO/LO dietary treatment did not alter either total cytochromes

TABLE 4

Influence of Different Sources of Dietary Fats on Total P450 and the Activities of Microsomal Enzymes in Livers of Pregnant Mice[a]

	Dietary fat		
	HCO	SAFF	CLO/LO
Total P450 content, nmol/mg protein	0.47 ± 0.05	0.44 ± 0.05	0.35 ± 0.02
Ethoxyresorufin deethylase, pmol/(min•mg protein)	50 ± 5	43 ± 5	47 ± 3
7-Pentoxyresorufin *O*-dealkylase, pmol/(min•mg protein)	83 ± 13	64 ± 9	78 ± 11

[a]Pregnant mice were fed diets containing 2 wt% safflower oil (SAFF) and 10 wt% of either hydrogenated coconut oil (HCO), SAFF, or a cod liver oil/linseed oil mixture (CLO/LO) for three weeks prior to impregnation and until day 12 of gestation. Values are expressed as means ± SEM of ten mice in each group.

P450 content nor the activities of cytochromes P450 isozymes known to bioactivate DPH, it is unlikely that differences in maternal cytochromes P450 metabolism of DPH could account for the observed protective effects of the CLO/LO diet. The similarity of liver cytochromes P450 content and activities between the CLO/LO and SAFF diets is in agreement with other studies demonstrating that dietary 18:3n-3, 20:5n-3 and 22:6n-3 are similarly effective to dietary 18:2n-6 and 20:4n-6 in increasing the content and activity of the liver microsomal cytochromes P450 mixed function oxidase system (35). Somewhat surprising was the lack of differences observed between the HCO diet and the dietary PUFA treatments as previous work has indicated that an elevation of PUFA intake increases cytochromes P450 activities (34,35). However, as a result of variables such as hormonal changes during pregnancy, inducibilities of cytochromes P450 between the pregnant and non-pregnant state may be altered (36).

In summary, the results reported herein provide support for the hypothesis of the involvement of embryonic prostaglandin synthase in the bioactivation of DPH as a mechanism of DPH teratogenicity. Additionally, the role of gestational delay in palatal development in decreasing DPH-induced cleft palate incidence in n-3 fatty acid fed mice is suggested and requires further study. These observations do not discount a contribution from maternal enzymatic pathways of phenytoin metabolism for the decreased sensitivity of n-3 fatty acid-fed mice to DPH-induced cleft palate. Further research is needed to investigate the possibility that differences in distribution, conjugation and/or excretion pathways of DPH induced by n-3 diets may alter the plasma half-life and embryonic exposure to the drug. Since the final teratological endpoints of DPH toxicity depend on a balance among pathways involving bioactivation, detoxification and fetal cell repair, the protective effects of dietary n-3 fatty acids may result from a combination of effects exerted at a number of these metabolic steps.

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Plasma Lipids and Fatty Acids in Urbanized Bushmen, Hereros and Kavangos of Southern Africa (Namibia)

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Thirty-nine urbanized ethnic Namibian people comprising 21 Bushmen (semi-urbanized), 7 Hereros and 11 Kavangos were assessed for plasma lipids and fatty acid (FA) composition. Total cholesterol and triacylglycerol concentrations were measured by enzymatic methods, and neutral lipid FA composition by gas-liquid chromatography. The results demonstrated that while total cholesterol concentrations were not significantly different, significant differences in triacylglycerol concentrations ($P < 0.05$) were seen between Bushmen and Kavangos. By comparing Bushmen with Hereros and Kavangos, significant differences between Bushmen and Kavangos were also observed in plasma triacylglycerol FA compositions, particularly 16:0 (32.73% *vs.* 25.05%), 16:1n-7 (7.00% *vs.* 5.06%), 18:2n-6 (9.30% *vs.* 22.25%) and 20:3n-6 (0.12% *vs.* 0.48%), while Kavangos had higher 20:4n-6 levels than Hereros (1.44% *vs.* 2.00%). In plasma cholesteryl esters, Bushmen were significantly different from Kavangos in 16:1n-7 (8.85% *vs.* 4.93%), 18:1n-9 (32.06% *vs.* 23.07%) and 20:4n-6 (6.91% *vs.* 10.00%). Significant differences were also observed between Bushmen and Hereros in 18:0 (1.08% *vs.* 1.29%) and 18:2n-6 (35.68% *vs.* 45.50%). The FA of Namibian groups were also compared with South African reference groups comprising urbanized whites and Xhosas and rural Vendas. The differences in blood lipid values can be explained primarily by excessive alcohol consumption. These results suggest that semi-urbanized Bushmen have changed their diets under urbanized conditions which may increase their risk of coronary heart disease.

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Very little information is available on the plasma lipids and fatty acids (FA) in the different ethnic groups of Namibia, especially on Bushmen (1,2). Various studies have demonstrated that the plasma FA compositions, especially those of plasma cholesteryl esters (CE), reflect the dietary FA intake (3–6). Urbanization increases the risk of all groups to develop coronary heart disease (CHD), presumably due to changes in their dietary food intake (5–8), *e.g.*, increased alcohol consumption and a decreased dietary intake of vegetable fat. An increased mortality rate from CHD may endanger the existence of Bushmen in modern society (1).

We performed this study to examine the lipids and FA in semi-urbanized Bushmen *per se*, with comparison to Hereros and Kavangos of the same area, and white, Xhosa and Venda South African reference groups in order to investigate the extent of these dietary changes. White South Africans with a high incidence of CHD followed a typical Western diet with a high fat, low polyunsaturated to saturated fatty acids (P/S) ratio and low carbohydrate intake. The urbanized Xhosas are rapidly adopting a Western lifestyle which increases the risk of developing CHD. Rural Vendas consume a traditional diet of maize meal supplemented with mopanie worms.

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Abbreviations: AA, arachidonic acid; CE, cholesteryl esters; CHD, coronary heart disease; DGLA, dihomo-gammalinolenic acid; EPA, eicosapentaenoic acid; FA, fatty acids; GLC, gas-liquid chromatography; LA, linoleic acid; OA, oleic acid; P/S, polyunsaturated to saturated fatty acid ratio; TAG, triacylglycerol; TC, total cholesterol; TLC, thin-layer chromatography.

MATERIALS AND METHODS

A total of 39 urbanized ethnic Namibian people, in the Ojinene district of east Hereroland, comprising 21 Bushmen, 7 Hereros and 11 Kavangos with average ages of 29, 33 and 41, respectively, were assessed for plasma lipids and FA compositions. The Bushmen studied were no longer purely nomadic; most of them live for variable times in association with Herero villages, but tend to migrate from one place to another depending upon work and food availability. They are accordingly very much "in transition." Anthropometric measurements and dietary intake on the same study individuals have been reported previously by O'Keefe and Lavender (9). Approval to conduct a study on these ethnic groups was originally obtained from the local administrator or village headman. After an interview, informed consent was obtained from the subjects before blood samples were taken with the assistance of local district nurses. All participants were adults older than 18 years.

All blood samples were taken in the early morning, in either fasting or semi-fasting (light maize meal and water) conditions. Blood samples were taken in heparinized containers, and separated plasma samples were deep-frozen until analysis could be carried out, which was always within two weeks. Total cholesterol (TC) and triacylglycerol (TAG) concentrations were determined enzymatically (Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany; CHOD-PAP and GPO-PAP kits, respectively). Neutral lipids were separated by thin-layer chromatography (TLC) and analyzed by gas-liquid chromatography (GLC) as described previously (10). The significance of these results was evaluated using the nonparametric Kruskal-Wallis test to compare group means statistically at the 5% level. Although mean values were used, median values may be a better estimate of the true location of the mean. Robust standard deviations (SD) were used as an improvement on the usual SD calculated for the subgroup specifically, due to the small sample size of the Herero and Kavango groups. FA results are compared with urbanized whites and blacks (Xhosas), and rural blacks (Vendas) in South Africa (unpublished data).

RESULTS

The findings of the trial are summarized in Tables 1, 2 and 3. It should be noted that Table 1 includes data for only 13 Bushmen, 4 Hereros and 10 Kavangos, compared with the data of 21 Bushmen, 7 Hereros and 11 Kevangos in Tables 2 and 3. This was due to insufficient sample size

TABLE 1

Plasma Total Cholesterol and Triacylglycerol Concentrations in Urbanized Ethnic Groups (in mmol/L)[a]

					Reference groups		
Lipid	Bushmen (n = 13) mean (SD)	Hereros (n = 4) mean (SD*)	Kavangos (n = 10) mean (SD*)	Statistical difference $P < 0.05$	SAU Whites (n = 50) mean (SD)	SAU Xhosas (n = 50) mean (SD)	SAR Vendas (n = 61) mean (SD)
TC	3.71 (1.03)	4.21 (1.18)	3.61 (1.18)	K B H	6.24 (1.14)	4.08 (0.89)	4.23 (0.84)
TAG	2.01 (1.18)	1.35 (0.79)	1.06 (0.79)	K H B	2.01 (1.21)	1.19 (0.75)	1.26 (0.72)

[a]SD, standard deviation; SD*, robust standard deviation; SAU, South African urbanized; SAR, South African rural; non-significance is indicated by a connecting bar.

TABLE 2

Plasma Triacylglycerol Percentage Fatty Acid Composition (weight percent) in Urbanized Ethnic Groups[a]

					Reference groups		
Fatty acid	Bushmen (n = 21) mean (SD)	Hereros (n = 7) mean (SD*)	Kavangos (n = 11) mean (SD*)	Statistical difference $P < 0.05$	SAU Whites (n = 50) mean (SD)	SAU Xhosas (n = 50) mean (SD)	SAR Vendas (n = 61) mean (SD)
16:0	32.73 (5.23)	30.33 (6.06)	25.05 (6.06)	K H B	23.19 (3.69)	25.07 (4.35)	29.93 (5.50)
16:1	7.00 (2.19)	5.41 (2.36)	5.06 (2.36)	K H B	4.67 (1.21)	4.22 (1.65	5.70 (1.62)
18:0	5.94 (1.98)	5.85 (1.76)	3.68 (1.76)	K H B	5.03 (1.27)	6.71 (1.72)	3.82 (1.36)
18:1	43.38 (4.73)	47.60 (4.77)	41.34 (4.77)	K B H	39.02 (4.36)	39.63 (4.60)	40.96 (4.94)
18:2	9.30 (6.56)	9.37 (10.18)	22.25 (10.18)	B H K	24.43 (6.07)	20.70 (6.04)	17.79 (6.84)
20:3	0.12 (0.18)	0.18 (0.28)	0.48 (0.28)	B H K	0.48 (0.41)	0.42 (0.16)	ND
20:4	1.44 (0.92)	1.07 (0.81)	2.00 (0.81)	H B K	2.38 (1.40)	2.59 (0.95)	1.72 (0.64)
20:5	0.07 (0.12)	0.19 (0.17)	0.14 (0.17)	B K H	0.81 (0.78)	0.66 (0.58)	0.64 (0.15)

[a]SD, standard deviation; SD*, robust standard deviation; SAU, South African urbanized; SAR, South African rural; ND, not detected; non-significance is indicated by a connecting bar.

TABLE 3

Plasma Cholesteryl Ester Percentage Fatty Acid Composition (weight percent) in Urbanized Ethnic Groups[a]

					Reference groups		
Fatty acid	Bushmen (n = 21) mean (SD)	Hereros (n = 7) mean (SD*)	Kavangos (n = 11) mean (SD*)	Statistical difference $P < 0.05$	SAU Whites (n = 50) mean (SD)	SAU Xhosas (n = 50) mean (SD)	SAR Vendas (n = 61) mean (SD)
16:0	14.15 (4.42)	11.86 (2.54)	14.84 (2.54)	H B K	10.79 (1.42)	11.13 (1.62)	12.03 (1.78)
16:1	8.85 (3.93)	4.74 (3.40)	4.93 (3.40)	K H B	3.54 (1.26)	3.24 (1.80)	4.92 (2.19)
18:0	1.08 (0.67)	1.29 (0.37)	1.14 (0.37)	B K H	1.35 (0.32)	1.64 (0.50)	0.53 (0.32)
18:1	32.06 (6.88)	27.45 (7.35)	23.07 (7.35)	K H B	19.12 (2.22)	24.01 (4.79)	26.81 (4.02)
18:2	35.68 (13.08)	45.08 (11.77)	45.50 (11.77)	B K H	56.14 (5.11)	50.54 (6.75)	46.92 (5.11)
20:3	0.65 (0.64)	0.52 (0.49)	0.21 (0.49)	K H B	0.50 (0.29)	0.65 (0.22)	ND
20:4	6.91 (2.99)	8.48 (2.63)	10.00 (2.63)	B H K	6.63 (1.36)	7.68 (1.86)	8.63 (2.66)
20:5	0.62 (0.54)	0.57 (0.38)	0.31 (0.38)	K H B	1.92 (1.13)	1.11 (0.58)	0.17 (0.27)

[a]SD, standard deviation; SD*, robust standard deviation; SAU, South African urbanized; SAR, South African rural; ND, not detected; non-significance is indicated by a connecting bar.

to analyze the lipids of all the 39 participants, as priority was given to fatty acid analyses.

Statistically significant differences were found for TAG ($P < 0.05$) between Bushmen and Kavangos. Interestingly, Bushmen had the same TAG levels as the South African reference whites (Table 1). In neutral lipids the most important differences can be seen in plasma TAG percentage (TAG%) linoleic acid levels (LA; 18:2n-6), which were significantly higher in Kavangos compared to Bushmen and Hereros ($P < 0.05$) with a concomitant reduction of stearic acid (18:0). Bushmen had significantly higher ($P < 0.05$) palmitic acid (16:0) and palmitoleic acid (16:1n-7) and lower dihomo-gammalinolenic acid (DGLA; 20:3n-6) levels than Kavangos, while Kavangos also had significantly higher arachidonic acid (AA; 20:4n-6) levels than Hereros. Compared to the South African reference groups comprising urbanized whites and blacks (Xhosas) and rural blacks (Vendas), Vendas appear to have plasma TAG% palmitic acid levels similar to those of Bushmen and Hereros, while urban Xhosas and Kavangos have similar TAG% FA levels. Whites, however, have the highest LA levels of all the groups, with the Namibian groups all having higher oleic acid (OA; 18:1n-9) levels than any seen in the reference groups. Only Vendas have AA levels which are less than those of the Kavangos. In contrast, the reference groups have much higher eicosapentaenoic acid (EPA) levels than seen in the Namibian groups (Table 2).

Bushmen had significantly higher ($P < 0.05$) palmitoleic acid and OA levels and lower AA levels than Kavangos in plasma percentage cholesteryl esters (CE%), where individual FA were expressed as percentages of the total area of all FA peaks. Bushmen also had significantly lower ($P < 0.05$) stearic acid and LA levels than Hereros. Compared to the South African reference groups, Xhosas and Vendas have CE% composition palmitic acid levels which are similar to that of the Hereros, while whites seem to have the lowest levels of all groups. Palmitoleic acids are even lower in whites and Xhosas when compared with the levels of Hereros and Kavangos. Xhosas also have the highest stearic acid levels while Vendas have the lowest of all groups. Whites have the lowest OA levels of all groups, while Xhosas and Vendas have OA levels comparable to those of the Kavangos and Hereros, respectively. Both whites and Xhosas have higher LA levels than seen in all the Namibian groups, with whites having the highest. Although whites and Bushmen have similar AA levels, whites and Xhosas seem to have generally higher AA levels than seen in the other Namibian groups (Table 3). It should be noted that the reference whites also have the highest eicosapentaenoic acid (EPA; 20:5n-3)/AA ratio (0.29), compared to Xhosas (0.14), Bushmen (0.09), Hereros (0.07) and Kavangos (0.03). Bushmen also have a lower P/S ratio in their CE% FA than whites, 5.57 *vs.* 7.23, respectively (Table 3). Unfortunately, no phospholipid levels were measured to support the CE% FA; this could have provided a more specific interpretation of the habitual dietary intake of each group.

DISCUSSION

Bushmen, the original inhabitants of southern Africa, are scattered over large areas of northeast Namibia. Their diet varies dramatically and depends primarily on availability, with the only consistent component being maize meal. Home brewed beer consumption (2–4 liters per day) provides a daily energy intake from alcohol of 740–1480 kcal and contributes 156–312 grams of carbohydrate. Little or no vegetables and fruit are eaten. The Kavangos live in the far north and rely mainly on agriculture. Their staple diet consists of "African millet" (*mahongo*), a whole grain product, supplemented by fish from the river. Their diet is well balanced and reasonably constant. Hereros, the major black group, occupy the central areas of the territory. They mainly consume meat and dairy products with a staple diet of refined maize meal, 2–3 times daily. Hereros tend to consume more vegetables, fruits and fresh milk during the short rainy season.

An interesting observation was made when comparing the data in Table 1. Bushmen had substantially higher mean TAG concentrations than Kavangos. While TAG concentrations for Kavangos and Hereros were similar to previous observations in rural groups (1,2), the values for Bushmen were nearly doubled. This could be ascribed, possibly, to excessive alcohol consumption which causes TAG accumulation in the liver (11). Whilst total caloric and animal protein intake is low (1,9), animal fat (lard) is used when available mainly for flavoring by mixing with maize meal. TC concentrations were similar in all groups, but the general tendency was lower than in the rural population (1) and lower compared to other groups (12–13).

This communication compares for the first time the neutral lipid FA compositions in these southern African groups. Groups were not separated according to sex, because no sex difference could be detected in studies by Holman *et al.* (14) and Manku *et al.* (15). The effect of age should have little influence on the CE% FA composition (16); accordingly, population CE% FA differences are most likely to be due to dietary differences (6). The high LA content in the plasma CE of Hereros and Kavangos, and concomitant decreases in palmitoleic acid in these groups compared to Bushmen, suggests that these groups consume relatively less animal products than do the Bushmen, because plasma CE FA are known to have highly significant associations with dietary fat consumption due to the preference to plasma CE as indicators of dietary fat compositions (4–6). These findings are in accordance with a study of Melchert *et al.* (17). The lower LA content in Bushmen may also indicate a reduced polyunsaturated fat intake by this group (18) and a relatively high saturated fat intake, because vegetable and fruit consumption is little to none at all, which could be expected to increase the risk for CHD or atherosclerosis (6–7,19). Only few of the rural Bushmen, living from hunting and gathering, survive today. They had high LA TAG% FA values of 25.9% compared with the 9.3% as measured in the participating group (2,9). Because the plasma CE% FA of Bushmen are similar to those of normal Italian subjects, this may be indicative of a similar dietary P/S ratio (3). The FA of the Kavangos and Hereros are similar to values for groups reported by other investigators (19–21). However, South African whites and Xhosas have higher LA levels (unpublished data).

Bushmen do, however, have the highest OA levels in their plasma CE. Compared to the other groups, this could protect Bushmen because OA tends to resist oxidative modifications of FA while it simultaneously reduces the concentration of low density lipoprotein cholesterol. The net effect could be a reduction in the progression of

atherosclerosis, which may explain in part the low incidence of CHD in Bushmen (22). The white control group had the lowest OA levels of all groups, which may be a contributory factor to the high incidence of CHD in this group (13). The white group also had higher CE P/S ratios than the Bushmen. Greenland Eskimos, well documented as having a reduced incidence of CHD (23), are characterized by having high OA levels and corresponding low CE P/S ratios (4,17). Lipid and FA comparisons of the different groups may oversimplify the risk elements of CHD and exclude the potential importance of genetic factors. However, very little information exists on the risk factors of the Namibian groups.

The high palmitoleic acid and OA levels in the CE% FA of Bushmen with concomitant low LA levels have typical FA patterns of chronic alcoholics after acute alcohol abuse (24), and may indicate excessive alcohol consumption. Both palmitoleic acid and OA are known to correlate positively with alcohol consumption, while LA correlates negatively with these variables. Alcohol is known to alter lipid metabolism (8), probably by stimulating hepatic FA biosynthesis or inhibition of Δ6 desaturase activity in these individuals (25).

Bushmen also had the lowest AA levels and the highest EPA/AA ratio of the Namibian groups. However, this is still considerably less than the EPA/AA ratio of the reference whites, who are known to be at risk of CHD (12). Although low ratios may increase the risk of developing CHD, the group of Bushmen studied had no clinical signs of CHD. This could perhaps become a problem with economic improvements resulting in increased total caloric intake. The change from their traditional lifestyle has resulted in a high incidence of malnutrition (9), especially reduced fat and protein stores (70% and 75%, respectively). The severe malnutrition is primarily responsible for the predominance of infectious disease in Bushmen, especially tuberculosis (9). One fact that was extremely clear was the high incidence of alcoholism. Urbanization of Bushmen may thus have dire consequences for their continued survival as an independent ethnic group in modern society (9,26).

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ARTICLE

Comparison of the Effect of the Amount and Degree of Unsaturation of Dietary Fat on Plasma Low Density Lipoproteins in Vervet Monkeys

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The effects of the degree of unsaturation and of the amount of dietary fat on low density lipoprotein (LDL) concentration and composition were determined in vervet monkeys. Diets with fat contents of 41, 31 and 18% energy, each with a low and a high polyunsaturated to saturated fatty acid ratio (P/S; 0.27–0.38 and 1.13–1.47) were fed to six female vervet monkeys for two months. Another six females were given a low fat, high P/S diet for the same period of time, to serve as a reference. The cholesterol contents of the diets were low (21–33 mg per day) and relatively constant. LDL cholesterol concentrations decreased significantly ($P \leq 0.01$) when the dietary fat content decreased from 31 to 18% of energy. The dietary P/S ratio only affected LDL cholesterol concentrations during moderate (31% of energy) fat intake, where LDL cholesterol increased ($P \leq 0.01$) with a decrease in dietary P/S. Substantial individual variations were observed in LDL cholesterol concentration responses to dietary fat changes. The changes in LDL cholesterol concentrations were the result of changes in the concentration of LDL particles, as the molecular composition did not differ significantly between dietary periods. The high density lipoprotein cholesterol and the plasma triacylglycerol concentrations were not influenced by the dietary fat changes. During the high P/S diets, the percentage of 18:2 (linoleic acid) increased ($P \leq 0.01$) and that of 18:1 (oleic acid) decreased ($P \leq 0.01$) in LDL esterified cholesterol, as compared to the low P/S diets. In adipose tissue triacylglycerol the percentage of 18:2 was three times higher ($P \leq 0.01$) during the high P/S diets than during the low P/S diets. A decrease in the amount of dietary fat (from 31 to 18% of energy) was associated with an increase in the percentage of 18:1 in LDL esterified cholesterol.

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Increased plasma (and especially low density lipoprotein) cholesterol concentration is a major risk factor for atherosclerosis (1). The cholesterol lowering effect of increasing the ratio of polyunsaturated to saturated fatty acids (P/S) in the diet have been demonstrated both in humans (2–7) and in non-human primates (8–11). Increasing the dietary P/S also is associated with increased percentages of 18:2 (linoleic acid) in adipose tissue and plasma lipids, which are negatively correlated with atherosclerosis (12–15). Dietary P/S used in these studies were often unrealistically high (P/S $\geq$ 2) (2,3,5,7,8,11).

Several human populations with low fat diets have low plasma cholesterol concentrations and risks for atherosclerosis (16–18). Usually these diets also have low saturated fat and cholesterol contents (17,19). In some experiments reductions in the amount of dietary fat were associated with decreases in plasma and low density lipoprotein (LDL) cholesterol concentrations (5,20,21), but not in others (4,6,22). Decreased high density lipoprotein (HDL) cholesterol (4,5, 19,21,22) and increased plasma triacylglycerol (TAG) concentrations (4–6,20), as well as decreased percentage of 18:2 in adipose tissue (23) (which have been reported during low fat diets), may increase the risk for atherosclerosis (1,14).

Alterations in the composition of LDL have been implicated in the etiology of atherosclerosis (15,24–26). Enlarged LDL particles with an increased esterified cholesterol content are associated with diet-induced hypercholesterolemia and atherosclerosis in non-human primates (15,27). The degree of unsaturation and the quantity of fat in the diet appear to be major factors in promoting these compositional changes in primates (2).

Only a few studies have compared the effects of increasing the P/S ratio and decreasing the amount of fat in the diet on lipoprotein metabolism (4–6,10,28,29). However, none of these studies determined the effect of a change in the degree of unsaturation of dietary fat during a moderate or low fat intake. Lipoprotein composition was not determined in any of these studies.

The aim of the present study was to determine both the combined and the independent effects of quantity and composition of dietary fat on plasma lipid and lipoprotein concentrations in female vervet (African green) monkeys. LDL molecular composition, as well as fatty acid composition of adipose tissue and LDL lipids, was selected for detailed investigation because of its documented association with atherosclerosis in this species (15,30).

MATERIALS AND METHODS

The study was approved by the Ethical Committee of the South African Medical Research Council.

The study group was comprised of 12 female vervet monkeys *(Ceropithecus aethiops)* between 1.5 and 4.5 years of age. The group was stratified for age and cholesterolemic response to a high fat (41% of energy), low polyunsaturated to saturated fatty acid ratio (P/S; 0.29) diet. The animals were then randomized into two groups of six animals each. The vervets were individually caged and kept in a controlled environment (31).

All 12 vervets were stabilized on a low fat (18% of

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Abbreviations: Apo, apolipoprotein; EC, esterified cholesterol; EDTA, ethylenediaminetetraacetic acid; FC, free (unesterified) cholesterol; HDL, high density lipoprotein; HF-HP, high fat, high P/S; HF-LP, high fat, low P/S; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDL-EC, low density lipoprotein esterified cholesterol; LF-HP, low fat, high P/S; LF-LP, low fat, low P/S; MF-HP, moderate fat, high P/S; MF-LP, moderate fat, low P/S; P/S, ratio of polyunsaturated to saturated fatty acids; TAG, triacylglycerol; TPL, total phospholipid; VLDL, very low density lipoprotein.

energy), high P/S (1.47) diet (LF-HP diet) for two months before the start of the experiment. The experimental group was fed a series of six diets which differed in the amount and/or type of dietary fat. The diets fed in consecutive periods were: high fat, low P/S diet (HF-LP diet); high fat, high P/S diet (HF-HP diet); moderate fat, high P/S diet (MF-HP diet); moderate fat, low P/S diet (MF-LP diet); low fat, low P/S diet (LF-LP diet) and the LF-HP diet. From the HF-LP to the LF-HP diet only one dietary variable changed after each dietary period. Each diet was fed for two months. Dietary studies on vervets showed that this period was sufficient for plasma cholesterol concentrations to stabilize after a dietary change (Smuts, C.M., and Kruger, M., unpublished data). The second group served as a reference group and was maintained on the LF-HP diet for the duration of the experiment.

The diets fed in the six sequential dietary periods consisted of food components common to human diets and were formulated from information contained in the NRIND Food Composition Tables (32). Food was provided in the form of baked patties. The major fat sources of the low P/S diets were beef, chicken (not in the low fat diet), butter and brick margarine; and in the high P/S diets they were beef, chicken, soft margarine (not in the high fat diet), brick margarine (only in the high fat diet) and sunflower oil. The other ingredients of the baked patties were skimmed milk powder, hake (white fish), egg, dried beans, rice, wheat bread meal (white or brown), sugar, syrup, potatoes, cabbage, carrots and bananas. Vitamin C (40 mg per vervet daily) was added after baking.

The diets were calculated to have the same energy, protein, fiber and cholesterol contents. The calculated fat content of the high, moderate and low fat diets was 40, 30 and 18% of energy, respectively, with a P/S of 0.25 for the low P/S diets and 1.50 for the high P/S diets, and a cholesterol content of 35 mg per monkey per day. The energy provided by carbohydrates compensated for changes in the energy provided by fat in the various diets. Total fat and cholesterol content and fatty acid composition of the diets were determined as described previously (30).

At the end of each dietary period, blood and adipose tissue samples were taken under ketamine anaesthesia (10 mg/kg body mass, intramuscular) with atropine premedication (0.05 mg/kg body mass, intramuscular). Blood samples were taken after a fasting period of at least 12 h. Blood was collected from the vervets into tubes containing ethylenediaminetetraacetic acid (EDTA; 1 mg/mL blood) and immediately centrifuged at 4°C to separate the plasma. Lipoprotein fractions were isolated by sequential ultracentrifugation. Very low density lipoprotein (VLDL) was isolated at a density of <1.006 g/mL, intermediate density lipoprotein (IDL) between 1.006 and 1.019 g/mL and LDL between 1.019 and 1.063 g/mL in a 40.3 Beckman (Palo Alto, CA) rotor at 40,000 rpm for 20 h at 10°C. The HDL fraction was isolated from the plasma by precipitating apolipoprotein B (apo B) containing lipoproteins with heparin-manganese chloride (33).

Total plasma and HDL cholesterol were measured using enzymatic methods (Boehringer Mannheim, Mannheim, Germany; CHOD-PAP method). Total and free cholesterol (FC) of the lipoprotein fractions isolated by ultracentrifugation were determined by an enzymatic iodide method (34,35). The enzymes used were cholesterol oxidase and esterase (Boehringer Mannheim). The esterified cholesterol concentration was obtained by subtraction. Plasma TAG concentration was determined enzymatically (Boehringer Mannheim). Apo B in LDL was assayed by endpoint laser nephelometry using a monospecific antibody to human apo B prepared in sheep (Boehringer Mannheim). All procedures were according to prescribed methods (36). The use of human anti-apo B antiserum against non-human apo B was validated as described (30).

Lipids in LDL and adipose tissue were extracted with chloroform/methanol (2:1, vol/vol) (37,38). Butylated hydroxytoluene was used as an antioxidant. Esterified cholesterol (EC), TAG and phospholipids were separated by thin-layer chromatography (30). The fatty acids of EC and TAG were transmethylated and analyzed by gas chromatography as described previously (30). Total phospholipids (TPL) were quantified by microchemical assays (39). The number of molecules of EC, FC TAG and TPL per LDL particle and LDL particle mass were calculated (30). Molecular weights (in g/mol) used in these calculations are 651 for EC, 387 for FC, 885.4 and 879.4 for TAG (for low and high P/S diets, respectively), 787 for TPL and 512,000 for apo B.

For the statistical analysis, the changes (delta values) within each individual vervet between consecutive dietary periods were calculated by taking differences. This was done for each vervet in both groups. By calculating these individual changes, each vervet is used as its own control. The two-sample Wilcoxon test was used to compare the delta values of the experimental and reference groups. The reference group values provided a measure of the non-dietary effect against which the changes in the experimental group could be assessed.

For comparison of the high and low P/S ratio diets, the mean of the values during the high and during the low P/S ratio diets, respectively, were calculated for each vervet in the experimental group. The differences (delta values) between these means were taken. These calculations also were done on the values of the corresponding periods in the reference group. The delta values of the two groups were compared by the two-sample Wilcoxon test. The effect of the amount of dietary fat was tested in the same way. Medians and range (minimum to maximum) were used for descriptive statistics. The median is a robust statistic for estimating the central location of a sample.

RESULTS

The content and composition of dietary fat in the six diets as determined by biochemical analyses are shown in Table 1. These results confirmed the calculated fat content of the diets. The analyzed P/S of the diets was higher (0.27–0.38) for the low P/S diets, and lower (1.13–1.47) for the high P/S diets than calculated from the food composition tables (0.25 and 1.50, respectively). The analyzed cholesterol contents of the diets were 61–92% of the estimated amount.

The baseline median body masses of the experimental (3.01 kg) and reference (2.65 kg) groups increased gradually over the experimental period by 0.35 kg and 0.45 kg, respectively. The lipid profiles of the two groups during the different dietary periods are shown in Table 2. Figure 1 shows the individual and median changes in plasma total and LDL cholesterol between consecutive dietary

TABLE 1

Diet Compositions

	Diet[a]					
	HF-LP	HF-HP	MF-HP	MF-LP	LF-LP	LF-HP
Energy (kJ/day)	1464	1517	1488	1469	1481	1468
(kcal/day)	350	362	355	351	354	351
Fat (% energy)[b,c]						
Total	40.0	42.1	31.4	31.0	18.4	18.1
Saturated	17.3	11.2	8.6	14.3	7.7	4.7
Monounsaturated	16.0	16.5	10.7	11.8	7.0	5.8
Polyunsaturated	5.0	12.6	10.8	3.9	2.9	6.9
18:2	3.7	12.0	10.4	3.1	2.2	6.1
P/S[c]	0.29	1.13	1.26	0.27	0.38	1.47
Protein (% energy)[b,d]	15.1	14.7	14.9	15.0	14.9	15.1
Carbohydrate (% energy)[b,d]	44.9	43.2	53.8	54.0	66.6	66.8
Cholesterol (mg/day)[c]	30	23	23	21	33	30
Fiber (g/day)[d]	5.2	5.3	5.3	4.5	5.3	5.2
Fatty acid composition (%)[c]						
12:0 (Lauric acid)	1.4	0	0	1.3	2.4	1.2
14:0 (Myristic acid)	5.9	0.6	1.2	6.3	0.4	0.3
16:0 (Palmitic acid)	24.2	17.0	17.2	26.3	24.6	17.2
18:0 (Stearic acid)	12.8	9.0	9.8	13.2	15.9	8.2
14:1 (Myristoleic acid)	1.1	1.2	0.2	1.1	0	0
16:1 (Palmitoleic acid)	3.2	1.6	2.1	3.4	2.3	2.5
18:1 (Oleic acid)	37.5	39.4	33.8	35.3	37.7	30.9
18:2 (Linoleic acid)	9.6	29.7	34.3	10.4	12.3	34.4
18:3 (Linolenic acid)	2.8	0.8	1.0	1.6	3.0	4.0
20:3 (Eicosatrienoic acid)	0.2	0	0.4	0.1	0.4	0.1
20:4 (Arachidonic acid)	0.3	0.4	0	0.4	0.3	0.1
22:5 (Docosapentaenoic acid)	0.1	0.1	0	0.2	0	0
22:6 (Docosahexaenoic acid)	0	0.2	0	0.3	0.4	0.2

[a]HF-LP, high fat, low P/S; HF-HP, high fat, high P/S; MF-HP, moderate fat, high P/S; MF-LP, moderate fat, low P/S; LF-LP, low fat, low P/S; LF-HP, low fat, high P/S.
[b]Energy conversion factor—fat, 37 kJ/g; protein, 17 kJ/g; and carbohydrate, 16 kJ/g (40).
[c]Calculated from analyzed values.
[d]Estimated from NRIND Food Composition Tables (32).

TABLE 2

The Influence of Dietary Fat Modifications on Plasma Lipid and Lipoprotein Concentrations[a]

Period	Diet[b]	Plasma cholesterol (mmol/L)[c]	LDL cholesterol (mmol/L)	HDL cholesterol (mmol/L)	Plasma TAG (mmol/L)[c]
Experimental group (n = 6)					
0	LF-HP	4.19 (2.86–5.18)	1.75 (0.72–2.75)	2.07 (1.67–2.69)	0.60 (0.43–0.78)
1	HF-LP	6.57 (4.69–7.77)	3.20 (1.70–4.51)	2.43 (1.76–2.94)	0.58 (0.46–0.82)
2	HF-HP	6.29 (3.25–7.30)	3.36 (1.33–4.97)	1.77 (1.23–2.83)	0.56 (0.50–1.45)
3	MF-HP	5.34 (3.20–6.35)[f]	3.14 (1.25–3.55)[d]	1.94 (1.60–2.92)	0.44 (0.28–1.67)
4	MF-LP	6.51 (3.75–6.54)[g]	3.28 (2.07–4.26)[e]	1.75 (1.63–2.92)	0.72 (0.46–1.55)
5	LF-LP	4.78 (2.86–5.55)[f]	2.22 (1.27–2.86)[d]	1.94 (1.47–2.88)	0.67 (0.41–0.89)
6	LF-HP	4.54 (2.68–4.85)	2.32 (1.12–3.47)	1.83 (1.39–2.45)	0.57 (0.39–0.71)
Reference group (n = 6)					
0	LF-HP	3.44 (2.55–4.95)	1.39 (0.85–2.84)	1.76 (1.54–2.27)	0.65 (0.54–0.79)
1	LF-HP	5.06 (4.46–5.99)	2.46 (2.12–3.82)	2.06 (1.45–2.53)	0.66 (0.49–0.87)
2	LF-HP	4.75 (3.66–5.73)	2.30 (2.02–2.73)	1.66 (1.23–2.44)	0.65 (0.27–0.92)
3	LF-HP	4.35 (3.41–5.49)	2.15 (1.59–3.18)	1.78 (1.39–2.48)	0.68 (0.38–0.90)
4	LP-HP	3.71 (3.30–5.44)	1.79 (1.49–2.74)	1.72 (1.24–2.35)	0.56 (0.44–0.80)
5	LF-HP	4.12 (3.78–5.23)	2.24 (1.65–3.13)	1.70 (1.56–2.05)	0.57 (0.36–0.67)
6	LF-HP	3.96 (3.80–4.56)	2.05 (1.80–2.36)	1.70 (1.41–2.14)	0.66 (0.51–0.82)

[a]Median with range in parentheses.
[b]LF, low fat; HF, high fat; MF, moderate fat; -HP, high P/S; -LP, low P/S.
[c]Cholesterol, 38.57 x mmol/L = x mg/dL; TAG, triacylglycerol, 87.62 y mmol/L = y mg/dL.
[d]and [e], and [f] and [g], Differed significantly ($P \leq 0.01$), when the changes in the experimental group were compared with the changes between corresponding periods in the reference group.

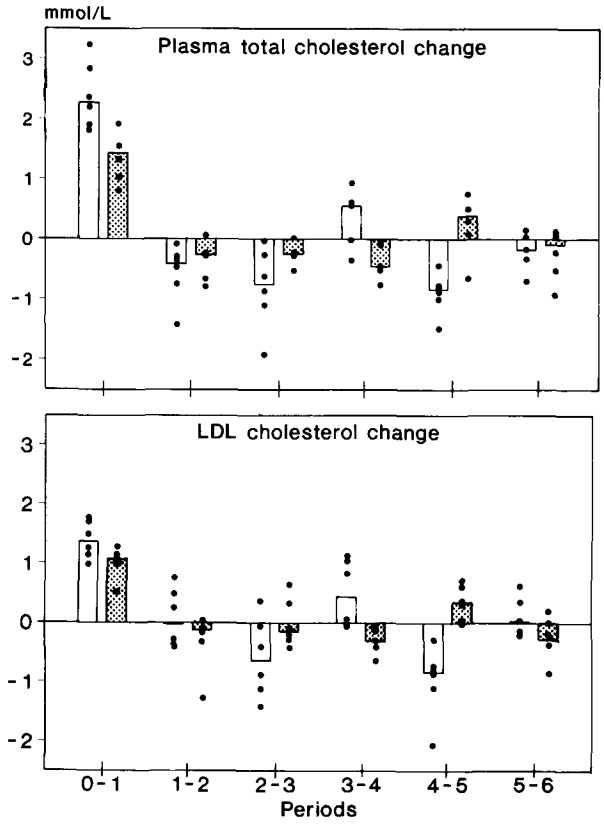

FIG. 1. Changes in individual (dot) and median (bar) plasma total and LDL cholesterol concentrations in experimental (open bar) and reference (dotted bar) groups between consecutive dietary periods. Dietary changes in the experimental group associated with the different periods were: 0–1, low fat, high P/S to high fat, low P/S; 1–2, high fat, low P/S to high P/S; 2–3, high fat, high P/S to moderate fat, high P/S; 3–4, moderate fat, high P/S to low P/S; 4–5, moderate fat, low P/S to low fat, low P/S; and 5–6, low fat, low P/S to high P/S.

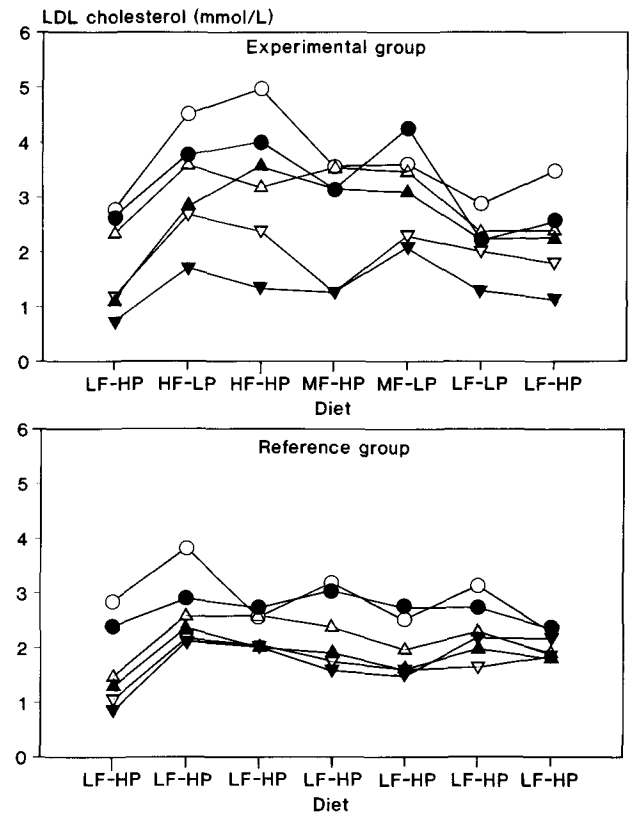

FIG. 2. Comparison of LDL cholesterol concentrations of individuals in experimental and reference groups. Diets: LF-HP, low fat, high P/S; HF-LP, high fat, low P/S; HF-HP, high fat, high P/S; MF-HP, moderate fat, high P/S; MF-LP, moderate fat, low P/S; and LF-LP, low fat, low P/S.

periods for the experimental and reference group. The median plasma total cholesterol concentrations of the experimental group decreased when the intake of fat with a low P/S was decreased from a moderate (31% of energy) intake to a low (18% of energy) intake ($P \leq 0.01$; Fig. 1, periods 4 to 5). The mean plasma total cholesterol concentrations were also lower during the low fat than during the moderate and high (41% of energy) fat dietary periods, when P/S was not taken into account ($P \leq 0.01$). The change in dietary P/S from 1.26 to 0.27 during the moderate fat intake was associated with a significant increase in the median plasma cholesterol concentration relative to the changes in the reference group (Fig. 1, periods 3 to 4). During the high and low fat diets the P/S did not have a significant effect on plasma total cholesterol (Fig. 1, periods 1 to 2 and 5 to 6, respectively). Plasma TAG and HDL cholesterol concentrations of the experimental group did not change significantly between dietary periods as compared to the reference group.

The individual and median values for LDL cholesterol concentrations of the experimental and reference groups are given in Figure 2 and Table 2, respectively. In the experimental group, changes in the LDL cholesterol concentrations were similar to those in plasma total cholesterol (Fig. 1). A decrease in the fat content of the diet from 31 to 18% of energy during the low dietary P/S was associated with decreases in the LDL cholesterol concentrations of all six vervets ($P \leq 0.01$; Fig. 2). Increasing the dietary P/S during the moderate fat intake resulted in lower LDL cholesterol concentrations in three of the vervets ($P \leq 0.01$; Fig. 2). The molecular composition (number of EC, FC, TAG and TPL molecules per molecule apo B) of LDL was not significantly affected by the amount or degree of unsaturation of fat in the diet (values not shown). This was also true for the calculated particle mass of LDL.

The percentages of 18:2 (linoleic acid) and 18:1 (oleic acid) in adipose tissue and LDL lipids are shown in Table 3, as well as changes in these parameters between consecutive dietary periods in Figure 3. An increased percentage of 18:2 in the dietary fat (high P/S diets, irrespective of the amount of dietary fat) was associated with increases in the percentages of 18:2 in adipose tissue and LDL lipids ($P \leq 0.01$; Tables 1 and 3). The percentage of 18:2 in adipose tissue TAG was about three times higher ($P \leq 0.01$) during the high P/S (P/S 1.13–1.47) than during the low P/S diets (P/S 0.27–0.38). In LDL-EC the percentage of 18:2 increased ($P \leq 0.01$), while that of 18:1 decreased ($P \leq 0.01$) during the high P/S diets. This situation was

TABLE 3

Effect of Dietary Fat Modifications on the Percentages of 18:2 and 18:1 in Adipose Tissue and LDL Lipids[a]

		18:2		18:1	
Period	Diet[b]	Adipose tissue TAG[c]	LDL CE[c]	Adipose tissue TAG	LDL CE[c]
Experimental group					
0	LF-HP	23.1 (20.3-25.0)	58.3 (53.9-65.0)	41.2 (39.4-46.3)	16.7 (15.4-21.0)
1	HF-LP	7.6 (6.2-8.3)	51.5 (50.2-54.3)	48.6 (41.0-50.2)	25.4 (23.3-27.0)
2	HF-HP	24.7 (24.0-27.5)	62.0 (53.0-67.4)	44.2 (43.6-45.1)	16.2 (14.7-18.8)
3	MF-HP	23.5 (23.1-26.1)	62.0 (58.3-66.0)	42.2 (40.3-44.0)	16.0 (14.4-18.5)
4	MF-LP	7.6 (7.3-8.6)	50.2 (47.8-54.1)	44.2 (42.8-46.2)	22.2 (20.4-25.5)[d]
5	LF-LP	6.1 (5.6-6.5)	45.7 (41.6-50.5)	48.1 (45.5-53.3)	29.3 (26.3-32.6)[e]
6	LF-HP	18.2 (11.9-20.2)	60.2 (58.5-63.2)	44.3 (42.0-48.1)	18.3 (16.5-20.2)
Reference group					
0	LF-HP	21.6 (20.0-28.1)	60.9 (58.1-67.0)	42.3 (36.3-45.4)	16.0 (14.7-20.7)
1	LF-HP	20.3 (15.6-25.2)	65.8 (59.0-68.1)	41.0 (38.8-45.0)	16.1 (14.8-18.1)
2	LF-HP	23.2 (16.6-26.1)	58.0 (54.9-60.9)	39.1 (35.4-40.8)	14.7 (13.8-18.4)
3	LF-HP	21.9 (18.6-29.6)	61.5 (57.5-64.4)	39.5 (38.1-46.9)	15.5 (14.9-17.3)
4	LF-HP	16.3 (15.4-17.3)	58.7 (51.3-60.0)	38.6 (36.9-43.1)	17.6 (15.0-21.1)
5	LF-HP	21.3 (19.0-27.1)	66.1 (61.3-68.5)	42.8 (38.8-45.4)	18.3 (16.0-19.6)
6	LF-HP	21.2 (16.2-23.3)	63.9 (59.5-66.5)	41.9 (39.0-45.5)	16.8 (14.3-19.6)

[a]Median with range in parentheses. LDL, low density lipoprotein.
[b]LF, low fat; HF, high fat; MF, moderate fat; -HP, high P/S; -LP, low P/S; TAG, triacylglycerol.
[c]Significant difference between high (-HP) and low P/S (-LP) diets ($P \leq 0.01$).
[d]and [e]Differed significantly ($P \leq 0.01$), when the changes in the experimental group were compared with the changes between corresponding periods in the reference group.

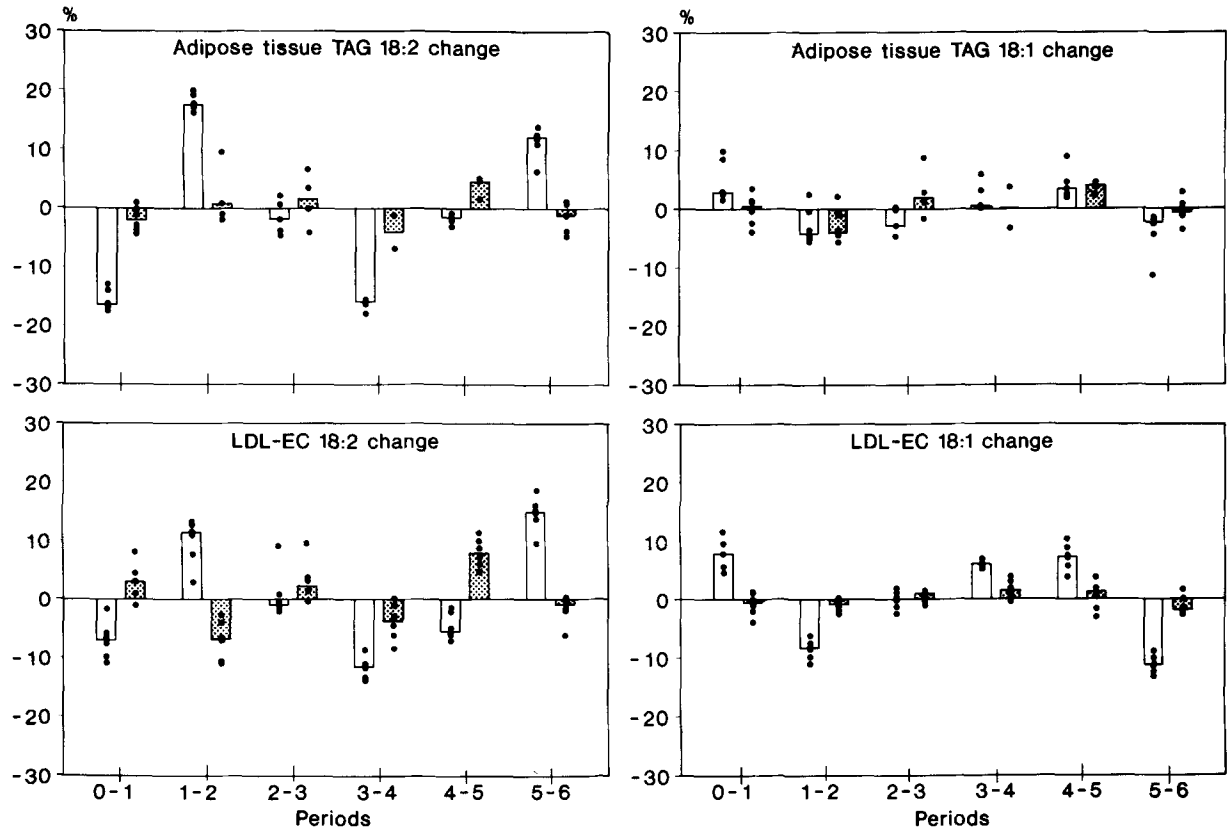

FIG. 3. Changes in individual (dot) and median (bar) percentages of 18:2 and 18:1 in adipose tissue triacylglycerol (TAG) and LDL esterified cholesterol (LDL-EC) in experimental (open bar) and reference (dotted bar) groups between consecutive dietary periods. Dietary changes in the experimental group associated with the different periods were: 0-1, low fat, high P/S to high fat, low P/S; 1-2, high fat, low P/S to high P/S; 2-3, high fat, high P/S to moderate fat, high P/S; 3-4, moderate fat, high P/S to low P/S; 4-5, moderate fat, low P/S to low fat, low P/S; and 5-6, low fat, low P/S to high P/S.

reversed with a change to a low P/S diet. The change in dietary fat content from 31 to 18% of energy was associated with an increase in the percentage of 18:1 in LDL-EC during a low dietary P/S ($P \leq 0.01$; Fig. 3, periods 4 to 5). The percentage of 18:2 in adipose tissue and LDL lipids tended to decrease when dietary fat contents were decreased, although not significantly. Neither changes in the degree of unsaturation nor the amount of dietary fat influenced the percentage of 18:1 in adipose tissue TAG or the percentage of 16:0 in adipose tissue TAG or LDL-EC (values not shown).

DISCUSSION

This study provides evidence that the amount of dietary fat had a greater influence on plasma cholesterol concentration than a moderate change in dietary P/S in vervets. Plasma cholesterol concentration tended to decrease with the reduction in dietary fat from 41 to 31%, while a greater, significant decrease ($P \leq 0.01$) was observed with the reduction from 31 to 18% of energy. The effects of dietary fat on plasma cholesterol were mainly through changes in LDL cholesterol concentrations. This agrees with reports for non-human primates where dietary fat contents were decreased from at least 27% to less than 7% of energy (10,28). In some studies on humans a reduction in dietary fat (from 40 to 27% of energy) was associated with decreases in plasma and LDL cholesterol concentrations in a five-week period (20,21). As in this study, Weisweiler *et al.* (6) found that a smaller change in greater quantities of dietary fat (from 42 to 32% of energy) did not significantly effect plasma cholesterol concentrations in humans. The lack of an effect of fat reduction in other studies (4,22) may be attributed to shorter dietary periods (≤3 weeks).

The significant increase in LDL cholesterol concentration with the decrease in P/S (from 1.26 to 0.27) during the moderate fat intake was smaller in effect than the decrease with the reduction in fat intake from 31 to 18%. The small increase (4%) in the median LDL cholesterol concentration with an increase in dietary P/S in the present study may be partly due to the fact that only female vervets were used. This is supported by the finding of Lewis and Taylor (41) that the decreases in the plasma cholesterol concentrations with an increase in dietary P/S were smaller in female African green monkeys as compared to the males.

The lack of an effect of dietary P/S changes during the high fat intake is in contrast to findings of other studies on African green monkeys (9,11). In these studies, however, changes in dietary P/S were greater (5- to 25-fold, compared to 4-fold), and cholesterol contents higher (0.4–0.8 mg/kcal, compared to 0.2 mg/kcal) than in the present study. The finding that an increase in dietary P/S does not further decrease plasma and LDL cholesterol concentrations during low fat intake is supported by the observations of Kuusi *et al.* (42) in humans during a fat intake of 23% of energy. However, during a fat intake of 25% of energy, Judd *et al.* (43) found that plasma total and LDL cholesterol concentrations decreased with a P/S increase of 0.3 to 1.0.

This study was not designed to compensate for any possible carryover effects. However, at the end of the experimental period the median plasma total cholesterol concentration of the experimental group was 8.4% higher than the baseline value. For the reference group the corresponding increase was 15.1%. This indicates that the effects of the different diets fed to the experimental group were not carried over to the last dietary period. The increases in plasma total and LDL cholesterol concentrations in the reference group in the first dietary period were probably due to non-dietary influences, *e.g.*, stress associated with the experimental procedure.

The vervets showed marked individual differences in LDL cholesterol concentration response to both the amount and the degree of unsaturation of fat in the diet. Individual reactions to a reduction in dietary fat (in humans, see refs. 29,44) and to changes in the dietary P/S (in non-human [9,45] and human primates [23,29,46–48]) have been reported previously. Genetic factors probably play a major role in the determination of an individual's sensitivity to changes in dietary factors (49). "Normal" fluctuations in an individual's plasma cholesterol concentration over time may obscure or minimize the response to dietary changes (50). In females, this fluctuation is partly the effect of periodic changes in the levels of their sex hormones (51). The influences of hormonal changes can be reduced by analyzing blood drawn at corresponding points during menstrual cycles. This would mean that experiments have to last longer.

The low fat diets were not associated with increased plasma TAG concentrations or decreased HDL cholesterol concentrations, as reported in studies on non-human (10) and human primates (4,5,22,42,52). A longer-term study on humans (53) has failed to produce an increase in plasma TAG concentration, indicating that this may be a transient effect of dietary fat reduction.

The strong effect of the P/S of the diet on the fatty acid composition of lipoprotein and adipose tissue lipids agrees with previously reported results in rhesus monkeys (8) and humans (3,54). This is especially true for 18:2 (linoleic acid) and 18:1 (oleic acid). In this study the percentage of 18:2 in the diet was reflected in LDL-EC and adipose tissue TAG levels within two months; in humans, however, it can take as long as three years before adipose tissue fatty acids reflect dietary fatty acids (23,55,56). The fast rate of these changes in vervets, which have a much lower percentage of body fat and a higher metabolic rate than humans, is probably due to a faster turnover of adipose tissue fatty acids (55). The observation that the median percentage of 18:1 in the adipose tissue TAG of the vervets was higher than in the dietary fat is indicative of a high rate of synthesis of 18:1 in this tissue. Fatty acid synthesis also has been reported to be more rapid in rat and in swine adipose tissue than in humans (57,58). The increased percentage of 18:1 in LDL-EC in periods of relatively low 18:2 intake was described previously in vervets (30) and in humans (3). This phenomenon may possibly be a compensatory mechanism to maintain fluidity in the LDL lipids. A significant positive correlation was found between atherosclerosis in non-human primates and the percentage of 18:1 in adipose tissue and LDL-EC (30), and the ratio of 18:1 to 18:2 in plasma EC (15). In this study decreasing the quantity of fat during a constant P/S was not associated with significant decreases in 18:2 in LDL or adipose tissue lipids, as was reported previously (23).

In theory an increased percentage of 18:2 in the core lipids of LDL particles can result in a reduction in the number of EC molecules per particle (2,3). The results of

the present study indicate that the EC content of LDL particles remained relatively unchanged during the six different dietary periods. LDL particle mass was also not influenced significantly by dietary fat changes, which supports previous findings both in vervets (30) and in humans (7,25,59). The loading of LDL with EC and FC, and increased LDL particle mass reported in these studies in non-human primates fed an atherogenic diet probably resulted from excessive cholesterol intake (24,26,27,60). The changes in the plasma and LDL cholesterol concentrations observed in the present study during dietary fat changes were therefore likely to be due to changes in the number of circulating LDL particles of relatively constant composition.

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Effects of Dietary Linseed Oil and Marine Oil on Lipid Peroxidation in Monkey Liver *in vivo* and *in vitro*

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Diets rich in linoleic acid (CO) from corn oil, or in linoleic acid and either α-linolenic acid (LO) based on linseed oil or n-3 fatty acids (MO) from menhaden oil were fed to male and female Cynomolgus monkeys for 15 wk. In the liver a 40% reduction of α-tocopherol occurred in the MO group relative to the CO and LO groups followed by increased formation of lipofuscin *in vivo*. A four-fold increase of α-tocopherol in the MO diet (MO + E) brought the level in the liver to that found with CO and LO. The increased peroxidation in the MO group in the liver phospholipids was associated with the replacement of 60% of the n-6 fatty acids by n-3 fatty acids from menhaden oil. Similar fatty acid profiles were found in groups fed MO and MO + E, respectively. Compared to the CO fed group, feeding α-linolenic acid only resulted in a slight incorporation of n-3 fatty acids in the liver membranes mainly due to a direct incorporation of α-linolenic acid. However, in monkeys fed menhaden oil more than 30% of the total fatty acids in the liver phospholipids were n-3 fatty acids. The various diets did not influence the activity of liver catalase (EC 1.11.1.6) nor superoxide dismutase (EC 1.15.1.1), but glutathione-peroxidase activity (EC 1.11.1.9) was higher in monkeys fed the MO diet. The catalase activity in females was 20% higher than in males. In an *in vitro* assay, liver microsomes from monkeys fed the MO diet or the MO diet supplemented with tocopherol produced similar amounts of thiobarbituric acid reactive substances and at a much higher rate than microsomes from the CO and LO groups. It appeared that α-tocopherol did not protect long-chain n-3 C_{20} and C_{22} fatty acids as well as n-6 fatty acids against peroxidation. The present data showed that monkeys were not fully able to compensate for increased peroxidative stress but a four-fold supplement of vitamin E to the diets reduced the oxidation.

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Diets rich in n-3 fatty acids have been shown to increase unsaturation in the membrane phospholipids of rat heart, liver and kidney (1,2) and of erythrocytes of cynomonkeys (3). Yet, little is known how the cells respond to the resulting increase in peroxidative stress. There is also only little information on the effects of dietary, highly unsaturated fatty acids on tissue lipid composition in primates in general.

The present study was undertaken to examine the effects of highly unsaturated dietary fats such as linseed oil and menhaden oil on the fatty acid profiles of liver phospholipids in monkeys. We further examined whether the monkey compensates for the increased oxidative stress by an induction of the three enzymes: catalase, superoxide dismutase (SOD) and glutathione-peroxidase (GSH-Px), which are known to remove the oxygen species believed to initiate lipid peroxidation (4). Furthermore, the ability of α-tocopherol to inhibit lipid peroxidation was examined.

MATERIALS AND METHODS

Four groups of Cynomolgus monkeys *(Macaca fascicularis)*, five males and five females in each group, were fed diets containing 15% (w/w) fat for 15 wk as previously described (3). The diets were: Diet 1, fat consisting of corn oil (Mazola Oil, Ottawa, Canada) and lard (Canada Packers, Toronto, Canada) designated corn oil (CO) diet; Diet 2, fat consisting of lard and linseed oil (Maple Leaf Monarch, Toronto, Canada) designated linseed oil (LO) diet; Diet 3, fat consisting of corn oil, lard and menhaden oil (Zapata Haynie Corp., Reedville, VA) designated menhaden oil (MO) diet; Diet 4, same as MO diet, supplemented with *all-rac* α-tocopheryl acetate and designated MO + E diet (Table 1). According to the manufacturer the menhaden oil contained 50 ppm free α-tocopherol and only 0.20% free fatty acids. In order to reduce the autoxidation to a minimum, all dietary oils were stored at −18°C under nitrogen, and the diets were freshly prepared every week and kept at 4°C as a gel. The monkeys were 6.5 ± 0.3 years old at the start of the feeding experiment.

After being fed the experimental diets for 15 wk, the monkeys were fasted for 18 h and anaesthetized with ketamin·HCl (Rogarsetic, Bristol Laboratories, Syracuse, NY). Blood was quickly drained from the *vena cava* by syringe. Liquid containing 10% (wt/vol) sodium citrate and 0.9% (wt/vol) NaCl was perfused through the body from a syringe in the left ventricle. The still-pumping heart caused an efficient perfusion of the whole body in a few minutes after which the liver was quickly removed. Portions of approximately 4 g were immediately frozen in liquid nitrogen and stored at −78°C for subsequent assays. Liver tissue (7 g) was immediately cooled in ice-cold 0.25 M sucrose, homogenized by a Potter-Elvehjem glass/Teflon homogenizer and adjusted with 0.25 M sucrose to a 10% (wt/vol) crude homogenate. Whole cells and cell debris were removed by centrifugation at 600 × *g* for 10 min. The supernatant (named liver homogenate) was kept on ice and enzyme, lipofuscin and protein assays were performed the day the animals were killed.

For the tocopherol analysis, 2 g of frozen liver was homogenized for 10 s in 4 mL of 0.25 M sucrose with a Polytron homogenizer (Kinematika AG, Littau, Switzerland) and adjusted to a 25% (wt/vol) homogenate. Following extraction, α- and γ-tocopherol was assayed by the high-performance liquid chromatography (HPLC) method of Thompson and Hatina (5) as described by Behrens and Madère (6).

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Abbreviations: CO, corn oil diet; GLC, gas-liquid chromatography; GSH-Px, glutathione peroxidase; HPLC, high-performance liquid chromatography; LO, linseed oil diet; MO, menhaden oil diet; MO + E, menhaden oil diet supplemented with *all-rac* α-tocopheryl acetate; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TLC, thin-layer chromatography.

TABLE 1

Fatty Acid Compositions of Dietary Fats and Content of α- and γ-Tocopherol[a]

Fatty acids	Dietary group			
	CO	LO	MO	MO + E
	(area percent)			
Saturated	39.7	39.7	43.9	43.1
Monoenoic	35.2	35.5	32.8	35.5
18:2n-6	24.7	11.8	10.6	10.8
18:3n-3	0.4	13.0	0.9	0.9
18:4n-3	—	—	1.8	1.9
20:5n-3	—	—	4.7	4.6
22:6n-3	—	—	2.8	2.4
Σ (n-6)	24.8	11.8	10.8	11.2
Σ (n-3)	0.4	13.0	10.9	10.2
	(μg/g diet)			
α-tocopherol	70.2	59.3	61.6	270.2
γ-tocopherol	59.0	9.4	8.5	6.5

[a]The dietary fats were the following: CO diet (36% corn oil and 64% lard); LO diet (34% linseed oil and 66% lard); MO diet (68% menhaden oil, 16% lard, and 16% corn oil); MO + E diet (68% menhaden oil, 16% lard, and 16% corn oil + an additional four-fold supplement of *all-rac* α-tocopheryl acetate). Fatty acid composition was determined by GLC after extraction and transmethylation of the lipids from the diets, and the tocopherols in the diets were measured by HPLC after saponification using external standards.

Lipofuscin was extracted from the liver homogenate and measured by fluorescence spectroscopy as described by Koster and Slee (7), and protein was assayed by the method of Lowry *et al.* (8).

The activity of the selenium containing GSH-Px (EC 1.11.1.9) was determined by the method of Flohé and Günzler (9). The activity of Cu,Zn-SOD (EC 1.15.1.1) was determined in the liver homogenate, diluted 10 times with 1% Tween-80 in 50 mM phosphate buffer, pH 7.0, using the procedure given by Prohaska (10). The assay for catalase (EC 1.11.1.6) was performed as previously described (11).

The lipids were extracted from the liver by the method of Bligh and Dyer (12), and the phospholipids were separated from the neutral lipids by thin-layer chromatography (TLC) (13), saponified, and methylated (14). The relative contents of the methyl esters were determined by gas-liquid chromatography (GLC) (15).

Microsomes for measurement of nonenzymatic peroxidation were prepared from a 25% (wt/vol) liver homogenate in 0.25 M sucrose by centrifugation at 20,000 × *g* for 15 min, followed by ultracentrifugation of the supernatant at 105,000 × *g* for 1 h. The pellet was resuspended in 50 mM Tris/HCl, 140 mM NaCl buffer pH 7.5 and kept on ice.

The microsomes were incubated with $FeCl_3$ and ascorbate to induce lipid peroxidation by the modified method of Hill and Burk (16). The final concentrations during the incubations were as follows: 12 μM $FeCl_3$, 2 mM ADP, 0.5 mM ascorbic acid, 50 mM Tris/HCl (pH 7.5), 140 mM NaCl, and microsomes corresponding to 0.5 mg protein/mL; the total volume was 1.5 mL. The mixture was incubated for 50 min at 37°C with vigorous shaking. After the incubation, aliquots were taken for tocopherol and TBA assays (17). For basal levels, Tris/HCl buffer replaced the ascorbic acid. Three incubations were carried out for each animal. Data were statistically analyzed by Student's *t*-test.

RESULTS

α- and γ-Tocopherol in monkey liver. Three of the diets contained similar levels of α-tocopherol; no differences in α-tocopherol contents were found in livers from monkeys fed either a diet rich in 18:2n-6 (CO diet) or rich in both 18:3n-3 and 18:2n-6 (LO diet), whereas feeding menhaden oil as a source of n-3 fatty acids (MO diet) reduced the level of α-tocopherol in the liver by 40% (Table 2). A four-fold higher dietary supplement of α-tocopherol given in the MO + E diet resulted in a liver content similar to the level found in the CO and LO groups.

The diets contained various amounts of γ-tocopherol of which corn oil, in particular, is a rich source (18). The level of γ-tocopherol in the liver reflected the level in the diets, the content in the CO group being approximately five times higher than in the three other groups. The levels of γ-tocopherol in the liver were in general lower in the MO and MO + E groups than in the LO group although these three groups received comparable dietary levels. No significant difference in either α- or γ-tocopherol between males and females given the same diets was observed. The presence of significant amounts of γ-tocopherol in the liver indicated that sufficient antioxidative capacity was present in the diets considering the superior antioxidant activity of γ-tocopherol compared to α-tocopherol in an oil (19). In addition the menhaden oil had a natural content of free α-tocopherol of 50 μg/g.

Lipofuscin. The content of extractable lipofuscin in liver homogenate was used as an indicator of *in vivo* lipid peroxidation. The level in the male MO group was three-fold higher than in the CO and LO groups (Table 3), whereas the level in monkeys fed the fish oil diet enriched in vitamin E, MO + E group, was neither significantly different from the MO nor the CO and LO groups. The same tendencies were found in the corresponding female groups, but the differences were not significant. No sex-related difference was observed in monkeys fed the same diets.

Catalase, superoxide dismutase and GSH-peroxidase activity in liver homogenate. The activities of catalase, SOD and GSH-Px were measured in a liver homogenate. The female monkeys had a 20% higher catalase activity than the males (significant only for CO and MO groups). The catalase activity was influenced neither by the dietary fat nor the vitamin E levels used in this experiment (Table 4).

The SOD activity seemed to be independent of the diets fed to the monkeys; no sex-related differences were observed.

The activity of the Se-containing GSH-Px was slightly increased in the groups fed the fish oil diets (MO and MO + E groups) but the increase was only significant in the female MO and MO + E groups, when compared with the CO group. The activity with LO fell between that obtained with CO and MO, without being significantly different from either of them. The activity found in the monkeys was of the same order of magnitude as found in human liver (36–62 nmol NADPH/min/mg protein (20)).

Fatty acid composition of phospholipids from liver. The fatty acid composition of liver phospholipids generally

TABLE 2

The α- and γ-Tocopherol Contents of Monkey Liver After Fifteen Weeks on the Experimental Diets[a]

	Dietary group			
	CO	LO	MO	MO + E
α-Tocopherol (μg/g liver)				
Males	21.0 ± 1.5[b]	20.0 ± 1.9[b]	12.4 ± 2.5[c]	26.7 ± 3.7[b]
Females	20.2 ± 1.7[b]	20.9 ± 3.9[b,c]	11.7 ± 1.0[c]	23.7 ± 2.9[b]
γ-Tocopherol (μg/g liver)				
Males	9.1 ± 0.7[b]	1.9 ± 0.3[c]	1.2 ± 0.3[c,d]	0.8 ± 0.1[d]
Females	8.4 ± 1.1[b]	2.1 ± 0.4[c]	0.9 ± 0.1[d]	0.8 ± 0.2[d]

[a]Contents of α- and γ-tocopherol determined in a 25% liver homogenate as measured by HPLC relative to external standards. Values represent mean ± SEM (n = 5). Values within each row not sharing a common superscript letter (b,c,d) are statistically different at $P < 0.05$. The dietary fats are as in Table 1.

TABLE 3

Content of Extractable Lipofuscin in Monkey Liver Homogenate[a]

	Dietary group			
	CO	LO	MO	MO + E
	(% T per mg protein)			
Males	7.7 ± 1.3[b]	7.4 ± 1.9[b]	23.4 ± 6.3[c]	13.5 ± 3.3[b,c]
Females	5.4 ± 0.4	10.0 ± 5.0	16.9 ± 5.0	8.0 ± 2.3

[a]Lipofuscin was measured by fluorescence spectroscopy; 50 mM sulfuric acid and 84.4 nM quinine hydrobromide was used for calibration at 0% and 100% transmittance, respectively. Excitation maximum: 344 nm; emission maximum: 540 nm. Values represent mean ± SEM (n = 5). Means in the same row not sharing a common superscript (b,c) are significantly different at $P < 0.05$. Dietary fats are as in Table 1.

TABLE 4

Activity of Catalase, Cu, Zn-Superoxide Dismutase (SOD) and Se-GSH-Peroxidase (GSH-Px) in Monkey Liver[a]

		Dietary group			
Enzyme	Sex	CO	LO	MO	MO + E
Catalase	M	32.5 ± 1.4[b]	33.4 ± 2.6	32.6 ± 2.1[b]	28.6 ± 2.7
	F	39.5 ± 2.1	39.6 ± 2.2	38.2 ± 0.9	34.9 ± 1.6
SOD	M	113 ± 6	115 ± 8	115 ± 6	104 ± 9
	F	118 ± 9[b,c]	132 ± 4[b]	120 ± 5[b,c]	112 ± 6[c]
GSH-Px	M	74 ± 8	78 ± 3	96 ± 8	89 ± 11
	F	71 ± 7[b]	92 ± 7[b,c]	99 ± 7[c]	89 ± 3[c]

[a]The activities were determined in a 10% liver homogenate after removal of cell debris by centrifugation at 600 × *g*. The units are: SOD (U/mg protein); GSH-Px (nmol NADPH/min/mg protein); catalase ($\Delta OD_{240\ nm}$/min/mg protein). The values represent mean ± SEM (n = 5). Means in a row not sharing a common superscript letter (b,c) are statistically different at $P < 0.05$. Dietary fats are as in Table 1.

[b]The activity in males (M) is significantly different ($P < 0.05$) from the activity in females (F) belonging to the same dietary group.

reflected the dietary fatty acids, the LO group being a noteworthy exception, as can be seen from Figure 1. The LO group fed the diet rich in both 18:3n-3 and 18:2n-6 differed only little in fatty acid composition from the CO group fed the high 18:2n-6 diet. The relative content of n-3 fatty acids was doubled in the LO group, but quantitatively the changes were small. The increase was mainly caused by incorporation of 18:3n-3, whereas the levels of some of its desaturation products, 20:5n-3, and 22:5n-3, were only slightly increased when compared to the CO group. No changes, however, were seen in the relative content of 22:6n-3.

The increase in n-3 fatty acids in the LO group was counterbalanced by a general decrease in the content of all the n-6 fatty acids. As a result the percentage of polyunsaturated fatty acids was similar in the CO and LO groups.

Feeding a diet rich in both 18:2n-6 and n-3 fatty acids

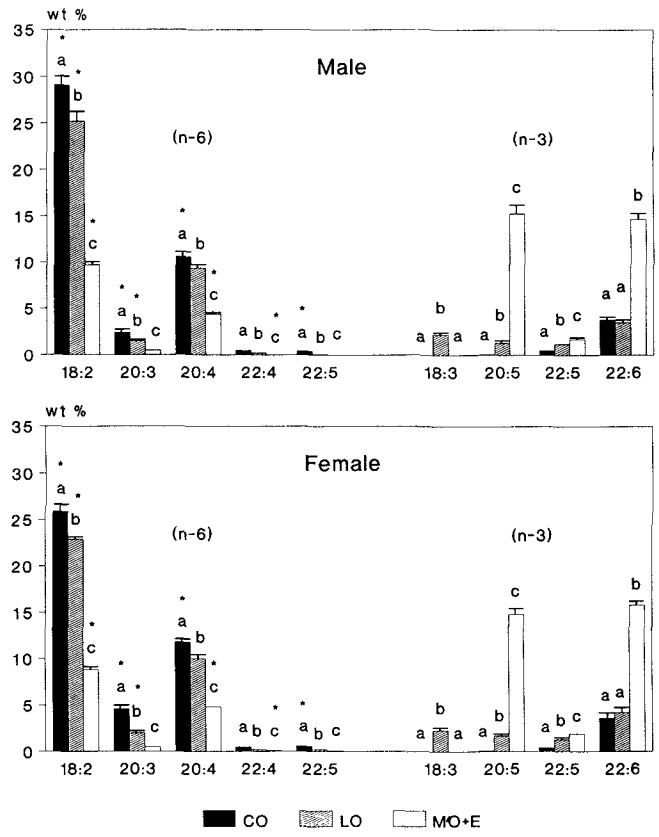

FIG. 1. Distribution of the major polyunsaturated fatty acids in liver phospholipids from monkeys fed the CO, LO and MO + E diets (area percentages determined by GLC). Each bar represents the mean ± SEM (n = 5), and bars for one fatty acid not sharing the same label are significantly different ($P < 0.05$); *, indicates a sex-related difference ($P < 0.05$).

from menhaden oil led to much more pronounced changes in the composition of the polyunsaturated fatty acids in the liver phospholipids. Compared to the CO group, the relative content of all the n-6 fatty acids was reduced by more than 60%, and was replaced particularly by 20:5n-3 and 22:6n-3. These highly unsaturated n-3 fatty acids were the most abundant polyunsaturated fatty acids in the liver phospholipids of monkeys fed the MO and MO + E diets. The addition of four times more α-tocopherol to the MO diet did not influence the fatty acid composition; therefore only the fatty acid composition for the CO, LO and MO + E groups are shown in Figure 1.

In general, similar tendencies were found in males and females, but a comparison between sexes fed the same diets showed that the females were lower in 18:2n-6 and higher in its desaturation products, 20:3n-6 and 20:4n-6. This sex-related difference was observed in all the dietary groups.

In vitro *lipid peroxidation in liver microsomes.* The microsomal contents of α-tocopherol in the CO, LO and MO + E groups were similar, whereas the content in the MO group was 10 times lower (Table 5).

After 50 min of incubation, all tocopherol levels decreased, with the CO group having a higher amount of α-tocopherol than the MO group; the levels in the LO and MO + E groups were not significantly different from the levels found in the CO or the MO groups. γ-Tocopherol levels in the microsomes were below the detection limit.

The basal TBARS level of the MO group was four times higher than that of the CO and LO groups, and twice as high as the MO + E group (difference not significant in the female MO + E group). Microsomes prepared from the MO + E group contained twice as much TBARS as the CO and LO groups (Table 5).

The monkeys fed the diets containing fish oil (MO and

TABLE 5

α-Tocopherol and TBARS in Monkey Liver Microsomes Before and After Fifty Minutes of Incubation with Fe^{3+} and Ascorbic Acid[a]

		Dietary group			
		CO	LO	MO	MO + E
α-Tocopherol (ng/mg protein)					
t_0	M	109 ± 15^{c}	89 ± 12^{c}	8 ± 2^{d}	120 ± 37^{c}
	F	130 ± 24^{c}	105 ± 24^{c}	13 ± 5^{d}	123 ± 29^{c}
t_{50}	M	56 ± 20^{c}	54 ± 26^{c,d}	4 ± 2^{d}	17 ± 15^{c,d}
	F	96 ± 18^{c}	35 ± 24^{c,d}	4 ± 3^{d}	54 ± 48^{c,d}
TBARS (nmol MDA/mg protein)[b]					
t_0	M	1.2 ± 0.2^{c}	1.0 ± 0.2^{c}	4.8 ± 0.8^{d}	2.6 ± 0.6^{e}
	F	1.2 ± 0.2^{c}	1.4 ± 0.2^{c}	5.2 ± 0.8^{d}	3.4 ± 0.6^{d}
t_{50}	M	22 ± 9^{c}	24 ± 10^{c}	106 ± 5^{d}	92 ± 9^{d}
	F	13 ± 7^{c}	39 ± 10^{d}	98 ± 4^{e}	72 ± 17^{d,e}

[a]The final incubation mixture contained 12 μM $FeCl_3$, 2 mM ADP, 0.5 mM ascorbic acid, 50 mM Tris/HCl (pH 7.5), 140 mM NaCl, and microsomes corresponding to 0.5 mg protein/mL. α-Tocopherol was determined by HPLC, and TBARS were measured in a TBA-assay; t_0 indicates the basal level in the microsomes, t_{50} indicates the level after 50 min incubation. Values represent mean ± SEM (n = 5). Means in a row not sharing a common superscript (c,d,e) are significantly different at $P < 0.05$. M, males, F, females. Dietary fats as in Table 1.

[b]TBARS relate to a MDA standard.

MO + E) produced more TBARS than the CO group (males four-fold higher and females eight-fold). TBARS production in the microsomes in the female LO group was significantly higher than in the CO group (three-fold) and lower than in the MO group (two-fold), although the CO, LO and MO diets contained the same amount of α-tocopherol. Compared to the MO + E group, the microsomes from the LO group produced 2-3 times less TBARS; however, the difference was only significant in the male groups.

It should be noted that the intra group variation for lipofuscin, α-tocopherol and TBARS contents was very large even though the experimental error was less than 5%. This reflected uniformly scattered data caused by biological variations between individuals.

DISCUSSION

Superoxide dismutase, catalase and GSH-Px participate in the cellular protection against lipid peroxidation *in vivo* by removing the potential initiators of the peroxidative processes, $O_2^{\cdot -}$, H_2O_2 and possibly other peroxides as well (4,21,22). In the present experiment only selenium containing GSH-Px seemed to be induced in the liver by the higher peroxidative stress associated with the intake of menhaden oil. However, the increase in activity could not fully compensate for the decrease in vitamin E in preventing the high accumulation of lipofuscin when fish oil was fed.

The very long-chain fatty acids, 20:1 and 22:1, are metabolized in the peroxisomes and induce higher peroxisomal β-oxidation and catalase activity in liver when fed to rats (23,24). Similarly, it was shown in a short term study that 20:5n-3 increased the peroxisomal β-oxidation in rat liver (25) and together with 22:6n-3 appears to be metabolized mainly by the peroxisomes (26). However, menhaden oil, rich in 20:5n-3 and 22:6n-3, did not seem to induce increased peroxisomal activity in the monkey liver when catalase activity was used as an indicator for the peroxisomal β-oxidation, even though the two n-3 fatty acids are likely to be better substrates for peroxisomal β-oxidation than 20:4n-6 as was shown in rats (27).

The higher catalase activity in females may indicate increased β-oxidation activity in the peroxisomes in females compared to males. A similar effect was observed in rats fed partially hydrogenated marine oil, where the increased peroxisomal β-oxidation in the female rats was accompanied by a lower accumulation of triglycerides in the heart compared to male rats fed the same diet (24). The increase in the content of 20:3n-6 and 20:4n-6 relative to 18:2n-6 seen in females did not affect the peroxidative status. Alternatively, the higher catalase activity prevented increased peroxidation.

The amount of α-tocopherol fed to the monkeys is considered to be adequate to fulfill the nutritional needs for vitamin E under normal conditions (28). In monkeys fed a diet similar to a typical Western European diet (the CO diet), the liver contents corresponded well to those observed in humans: 18.3 μg/g tissue and 9.2 μg/g tissue for α- and γ-tocopherol, respectively (29).

From the γ- to α-tocopherol ratio, it seemed that the absorption or retention of γ-tocopherol in the liver was less efficient than of α-tocopherol. It was the actual content of γ-tocopherol in the diet that influenced the γ-tocopherol content in the liver rather than the ratio of the two tocopherols. These results are in good accordance with the findings of Bieri and Evarts (30) and Peake and Bieri (31), who found that α- and γ-tocopherol were similarly absorbed but that γ-tocopherol was excreted more rapidly. Behrens and Madère (32), however, observed that a high amount of α-tocopherol affected the amount of γ-tocopherol absorbed, transported and taken up by tissues. The present data obtained on primates did not support the idea that additional dietary α-tocopherol affected the level of γ-tocopherol *in vivo*.

The levels of both antioxidants in the liver were reduced equally by the intake of the highly unsaturated menhaden oil as compared to monkeys fed diets rich in 18:3n-3. This suggests that although the biological antioxidant activity of γ-tocopherol is only about 30% of the activity of α-tocopherol (33,34), both tocopherols may play an important role as antioxidants *in vivo*. The reduced levels of tocopherols observed when menhaden oil was fed without vitamin E supplementation (MO group) were associated with increased lipid peroxidation, as indicated by the increased levels of lipofuscin and TBARS. This is in accordance with Mouri *et al.* (35), who demonstrated an increase in liver TBARS and a decrease in liver α-tocopherol when rats were fed increased amounts of cod liver oil. A four-fold increase in dietary α-tocopherol normalized the liver level when menhaden oil was fed, which is in good agreement with the findings of Meydani *et al.* (36) in mice. This, however, did not fully restore the antioxidative capacity of the liver *in vivo* (when lipofuscin is used as an indicator for peroxidation) and *in vitro* as measured by TBARS formation.

Feeding 18:3n-3 to the monkeys did not affect the peroxidative status of the liver *in vivo* compared with monkeys fed 18:2n-6. This can be explained by the moderate influence of the dietary 18:3n-3 on the fatty acid composition in liver phospholipids. Replacing the 18:3n-3 in the diets with the more unsaturated n-3 fatty acids of marine origin led to an extensive replacement of the less unsaturated n-6 fatty acids with the more unsaturated n-3 fatty acids, 20:5n-3 and 22:6n-3, followed by increased lipid peroxidation. This peroxidation could not be fully compensated for by a four-fold increase in dietary α-tocopherol. The liver phospholipid fatty acid pattern of monkeys fed menhaden oil with normal or high vitamin E content was similar, despite the increased level of one of the fatty acid peroxidation products, lipofuscin, found in the MO group. This could indicate that the damaged fatty acids were rapidly replaced by fatty acids from the diets or that the actual amount of polyunsaturated fatty acids lost due to lipid peroxidation is low compared to the total amount present in the membranes. However, it should be pointed out that the implication of lipofuscin formation is not clear.

In the *in vitro* experiment, even a small increase in the relative content of n-3 fatty acids after feeding linseed oil resulted in a higher risk of peroxidation under stressed conditions. Risk of peroxidation was further increased when the linseed oil was replaced by menhaden oil and a ten-fold increase of microsomal α-tocopherol could not reduce the peroxidation rate. It thus seems that α-tocopherol cannot protect membranes rich in n-3 fatty acids, especially with five or six double bonds, as efficient-

ly as membranes rich in n-6 fatty acids. This has also been observed in rat tissues (37-40).

The results from the Fe(III)/ascorbate incubation assay were in accordance with the results for lipofuscin formation; only the differences between the groups were much more pronounced in the *in vitro* assay. The non-enzymatic *in vitro* peroxidation in microsomes could as such be used as a measure of the peroxidation *in vivo* due to the dietary treatment.

Previous experiments have established that a four-fold supplement of α-tocopherol normalized the lipofuscin in rats fed marine oil (41). The present study indicated that such an additional supplement of α-tocopherol to primates did not fully prevent the peroxidative changes when menhaden oil was fed. This is probably caused by a higher level of polyunsaturated n-3 fatty acids incorporated in the liver from primates (32%) compared to rats (21%) after intake of marine oil, mainly due to a high incorporation of 20:5n-3 in the monkeys. This indicates that in primates a supplement of α-tocopherol beyond the levels used in this study is necessary in order to prevent peroxidation *in vivo*.

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Modulation of Cyclic Nucleotide Phosphodiesterase by Dietary Fats in Rat Heart

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Feeding oils of different fatty acid composition modifies the fatty acid composition of cardiac membrane phospholipids, thereby inducing changes in cardiac contractility and altering response of adenylate cyclase to catecholamines. In the present study, the effect of such dietary manipulations on cyclic nucleotide phosphodiesterase, which is involved in the control of cyclic nucleotide intracellular levels and in the control of cardiac contractility, was investigated. Rats were fed either a saturated fatty acid-enriched diet (8 weight percent [%] coconut oil + 2% sunflower oil), an n-6 fatty acid-enriched diet (10% sunflower oil) or an n-3 fatty acid-enriched diet (8% fish oil + 2% sunflower oil). The fatty acid composition of cardiac phospholipids, as well as the nonesterified fatty acid content of heart were markedly altered by the diets. The 18:2n-6 and 20:4n-6 content of cardiac phospholipids was markedly (−49%) depressed by fish oil as compared with sunflower oil feeding, but the nonesterified fatty acid level of heart membrane was lowest in coconut oil-fed rats. In addition, fish oil feeding more drastically depressed the n-6/n-3 fatty acid ratio in the nonesterified fatty acid pool than in cardiac phospholipids. Cyclic AMP phosphodiesterase activity was the lowest in both the particulate and soluble fractions of heart from rats fed sunflower oil, whereas cyclic GMP phosphodiesterase activity was not altered by the diets. Cyclic AMP phosphodiesterase activity was decreased by 18 and 12% in heart membranes of the sunflower oil group as compared to that of the coconut oil and fish oil groups, respectively. In heart cytosol, the activity decreased by 30% when compared with the activity of the coconut oil group. Additional *in vitro* experiments showed that polyunsaturated fatty acids were more potent inhibitors of cyclic AMP phosphodiesterase than saturated fatty acids. These results suggest that polyunsaturated fatty acid-enriched diets might decrease heart cyclic AMP phosphodiesterase activity by increasing non-esterified polyunsaturated fatty acids, especially those of the n-6 series, but more complex and indirect mechanisms are very likely to be involved.
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Several studies support the hypothesis that alterations in dietary lipids, particularly saturated *vs.* unsaturated fatty acids, can affect cardiac functions such as left ventricular work and coronary flow (1). Feeding sunflower oil as compared to saturated or standard diets has been shown to decrease the maximal response of adult rat atria to catecholamines (2) or the positive inotropic response of papillary muscles to calcium (3). Diets rich in polyunsaturated fatty acids (PUFA) not only decrease the occurrence of spontaneous tacchyarrythmias (3), but also reverse the high vulnerability to cardiac arrythmias previously induced in rats by feeding long-term a diet supplemented with saturated fatty acids (4). Dietary n-3 PUFA seem to offer greater protection than dietary n-6 fatty acids in several experimental models of myocardial ischemia (5,6). Diets supplemented with n-3 PUFA have been reported to reduce both the incidence and the severity of ventricular tacchycardia induced by myocardial ischemia and reperfusion (7), while those supplemented with n-6 PUFA have been shown to increase the sensitivity of rat myocardium to catecholamine-induced damage (8). Dietary fats can influence the lipid composition of plasma membranes in several tissues, including heart (9–11). These diet-induced alterations of the acyl constituents of membrane phospholipids can, in turn, induce functional changes in several ways. First, the acyl changes can alter the arachidonic acid content of membrane phospholipids and thus influence the production of bioactive eicosanoids. Second, as fatty acids are the major components of the hydrophobic core of the membrane bilayer, they can interact with and directly influence the functioning of some crucial membrane proteins involved in cellular communication and homeostatic processes such as receptors, ion channels and enzymes (12). Adenylate cyclase which controls cyclic nucleotide intracellular levels is one of the major enzymes involved in transmembrane signalling and, as such, is a key regulator of cell function, especially in the heart where cyclic nucleotides play a crucial role in regulating contractility (13). The β-adrenergic/adenylate cyclase system of cardiac membranes has been extensively studied. Alam *et al.* (14) have shown that both basal and stimulated adenylate cyclase activities were significantly increased in rats fed diets rich in n-3 PUFA. Although these results suggest that increasing cardiac PUFA content is associated with increased responsiveness of adenylate cyclase to adrenergic stimulation, not all investigators have come to this conclusion. Thus, Laustiola *et al.* (15) have reported that atria from rats fed n-3 PUFA-enriched diets had low basal and isoproterenol-stimulated accumulation of cyclic AMP, and Reibel *et al.* (16) have reported that dietary fish oil was associated with reduced inotropic responsiveness to α-, but not β-adrenoceptor stimulation when compared to n-6 fatty acids. However, the α_1-adrenoceptor-mediated phosphatidylinositol turnover rate does not seem to be affected by changes in the PUFA composition of membrane phospholipids in neonatal rat ventricular myocytes (17).

Intracellular cyclic nucleotide levels result from an equilibrium between their synthesis by the cyclases and their degradation by cyclic nucleotide phosphodiesterases (PDE, E.C. 3.1.4.17.). This enzyme system may play a crucial role in the control of cardiac functioning because

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Abbreviations: ANOVA, analysis of variance; BHT, butylated hydroxytoluene; CO, coconut oil; DMA, dimethylacetal; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EFA, essential fatty acid; FO, fish oil; IC_{50}, concentration of a drug which inhibited 50% of the enzymatic activity; PDE, cyclic nucleotide phosphodiesterase (E.C. 3.1.4.17); PMSF, phenylmethylsulfonylfluoride; PUFA, polyunsaturated fatty acid; SO, sunflower oil; TLC, thin-layer chromatography; Tris, trishydroxymethylaminomethane.

inhibitors of PDE are able to stimulate cardiac contractility and are used as potential therapeutic agents in the treatment of heart failure (18,19). Among the growing isoenzyme families of PDE, some of them, such as the calcium and calmodulin-stimulated isoform, can be activated by certain fatty acids and phospholipids (20) whereas others, such as the cyclic GMP-stimulated isoform, are rather inhibited, especially by unsaturated fatty acids (21). Until now, relatively few studies have addressed the question of the influence of dietary fats on PDE activity (22).

Therefore, the aim of the present study was to examine the influence of diets enriched with different fatty acids (saturated, n-6 and n-3) on PDE activity of rat heart. Diets were supplemented with a mixture of 8% coconut oil + 2% sunflower oil (relatively rich in saturated fatty acids and sufficient in essential fatty acids; EFA), 10% sunflower oil (relatively rich in n-6 fatty acids) or a mixture of 8% fish oil + 2% sunflower oil (relatively rich in n-3 fatty acids and sufficient in EFA) to induce changes in both the nonesterified fatty acid content of hearts and in the fatty acid composition of cardiac membrane phospholipids. The consequence of these changes on the enzyme activity was further examined.

MATERIALS AND METHODS

Animals and diets. O.F.A. rats (Iffa Credo, L'Arbresle, France) weighing 130–150 g were housed individually at 22 ± 1°C, given a commercial nonpurified diet and water *ad libitum*, and kept under a 12 h dark/light cycle for one week to acclimate the animals to laboratory conditions. Rats were then weighed and randomly divided into three groups of nine rats each so that the average weight per group was the same. They were fed semi-purified diets (differing only in the type of oils added) for a period of three weeks. The composition of the base semi-purified diet (Unité Rationnelle d'Alimentation, Villemaison, France), before the addition of 10 weight percent (%) oils, is summarized in Table 1. The coconut oil (CO) group was fed the base diet supplemented with 8% CO (source of saturated fat) mixed with 2% sunflower oil (SO) to prevent EFA deficiency. The SO group was fed the base diet supplemented with 10% SO as a source of n-6 fatty acid. The fish oil (FO) group was fed the base diet supplemented with 8% of purified concentrate of FO as a source of n-3 fatty acid, mixed with 2% SO. Commercial edible coconut and sunflower oils were obtained locally. Refined fish oil (Feniko) was purchased from Fournier Laboratories (Dijon, France). The fatty acid compositions of the diets are shown in Table 2. Fresh diets were prepared every four days by thoroughly mixing after the addition of oils. Daily amounts were stored in capped plastic tubes, under nitrogen, at −20°C, in the dark. Uneaten food was discarded and replaced with fresh diets stored as indicated above, daily. The absence of thiobarbituric acid reactive materials indicated that there was no oxidation of dietary fats.

Blood sampling. At the end of the feeding period, rats were killed by decapitation. Sacrifices were spread out over a period of three days (between 8–11 a.m.). Blood (4 mL) was collected immediately into plastic tubes that had been rinsed with heparin. Samples were then transferred to polypropylene tubes containing heparin (50 U/mL blood), gently mixed by inversion and centrifuged at 2,000 × *g* for 15 min. Plasma was removed and butylated hydroxytoluene (BHT) (5×10^{-5} M) was added before freezing at −80°C until further analyses.

Preparation of heart particulate and cytosolic fractions. Hearts were removed, perfused through aortic cannulae with physiological saline to remove blood, chopped into small pieces with scissors and homogenized in a glass-glass Potter-Elvejhem homogenizer with 5 vol (vol/wt) of 10 mM Tris-HCl buffer (pH 7.5) containing 0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was centrifuged at 1,000 × *g* for 10 min. The supernatant was then

TABLE 1

Composition of the Diets (g/100 g)[a]

	Coconut oil group	Sunflower oil group	Fish oil group
Delipidated casein[b]	20.25	20.25	20.25
Cornstarch + glucose	57.0	57.0	57.0
Cellulose	5.50	5.50	5.50
Mineral mix[c]	6.25	6.25	6.25
Vitamin mix[d]	1.0	1.0	1.0
Sunflower oil	2	10	2
Coconut oil	8	0	0
Fish oil	0	0	8

[a]Diets were isoenergetic and provided 14.6 Mcal/kg diet.

[b]Supplied in amino acids (mg/g diet): arginine, 8.5; cystine, 3; lysine, 17.4; methionine, 7.1; tryptophane, 5.0; and glycine, 1.0.

[c]Provided (g/kg diet): $CaHPO_4x2H_2O$, 30; KCl, 7; MgO, 3; $MgSO_4x7H_2O$, 3.5; Fe_2O_3, 0.2; $FeSO_4x7H_2O$, 0.35; $MnSO_4xH_2O$, 0.17; $CuSO_4x5H_2O$, 0.03; $ZnSO_4x7H_2O$, 0.14; $CoSO_4x7H_2O$, 0.0003; and stabilized KI, 0.0006.

[d]Provided (mg/kg diet): retinyl acetate, 6.93; cholecalciferol, 0.0025; thiamine HCl, 20; riboflavin, 15; dl-calcium pantothenate, 70; pyridoxine HCl, 10; meso-inositol, 150; cyanocobalamin, 0.05; dl-α-tocopheryl acetate, 170; menadione sodium bisulfite, 40; nicotinic acid, 100; choline, 1360; folic acid, 5; *p*-aminobenzoic acid, 50; and biotin, 0.3.

TABLE 2

Fatty Acid Composition of the Diets[a]

Fatty acids	8% Coconut oil + 2% sunflower oil	10% Sunflower oil	8% Fish oil + 2% sunflower oil
12:0	13.16		
14:0	25.08	0.33	0.76
16:0	17.96	8.12	4.49
16:1n-9		0.56	
16:1n-7			3.60
18:0	5.12	4.53	1.60
18:1n-9	14.25	18.51	10.80
18:1n-7		0.75	1.68
18:2n-6	24.43	67.20	19.60
18:3n-3			0.92
18:4n-3			5.15
20:1n-9			1.97
20:5n-3			29.34
22:5n-3			3.00
22:6n-3			17.10

[a]Values are reported as mol % of total fatty acids and represent an average of two determinations.

centrifuged at 105,000 × *g* for 1 h. The resultant pellet was resuspended in the above buffer without sucrose and centrifuged at 105,000 × *g* for 1 h. The first 105,000 × *g* of supernatant and the second 105,000 × *g* of pellet were used as cytosolic and membrane fractions and stored at −80°C until further analysis for both lipid composition and phosphodiesterase activity.

Enzyme assays. PDE activity of heart cytosolic and membrane fractions from rats fed the various diets was assayed by a two-step radioisotopic procedure modified according to Prigent *et al.* (23), with the following substrate conditions: 0.25 μM cyclic AMP, which reflects mainly the activity of the cyclic AMP-specific PDE isoforms; 0.25 μM cyclic GMP in the presence of either 1 mM EGTA or 100 U calmodulin plus 1 mM $CaCl_2$, which reflects the activity of the calmodulin-stimulated isoform. The cyclic GMP-stimulated PDE activity was measured with 5 μM cyclic AMP as substrate in the presence or absence of 5 μM cyclic GMP as the allosteric effector. Phosphodiesterase activities were expressed as pmol cyclic nucleotides hydrolyzed per min per mg protein. The inhibitory potency of several fatty acids was examined on the cytosolic cardiac enzyme prepared from rats fed a standard diet. The IC_{50} (concentration of a drug which inhibited 50% of the enzymatic activity) of fatty acids was calculated by plotting the percentage of enzymatic activity determined at 0.25 μM substrate concentration *vs.* the logarithmic concentration of the fatty acid. Fatty acids were dissolved in ethanol. Controls with vehicle were performed in each case. The 95% confidence limits for the IC_{50} values were determined by linear regression analysis according to Hubert (24).

Lipid extraction. Plasma and cytosolic, as well as particulate fractions from rat heart were extracted according to Bligh and Dyer (25). Heptadecanoic acid and diheptadecanoyl phosphatidylcholine were added to samples as internal standards. Briefly, 3 mL chloroform/methanol (1:2, vol/vol) containing 0.001% BHT were added to 1 mL samples and left at 4°C under N_2 for 10 min. Samples were then partitioned after addition of 1 mL saline and 1 mL chloroform. The lower phase was removed after centrifugation and the medium re-extracted with 2 mL chloroform. The combined organic phases were dried under N_2 stream.

Fatty acid analysis. For fatty acid analysis of total phospholipids and for nonesterified fatty acid determination, lipid samples were chromatographed on thin-layer chromatographic plates, developed with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol). Areas corresponding to phospholipids (at the origin) and free fatty acids (R_f 0.36) were scraped from the plate. Free fatty acid samples were methylated with diazomethane and phospholipid samples were directly transmethylated with boron trifluoride/methanol as described by Morrison and Smith (26). Fatty acid methyl esters and dimethylacetals (DMA) were quantified by gas-liquid chromatography (Girdel 3000, Lyon, France) using a 0.25 mm × 60 m SP 2380 capillary column (Supelco, Bellefonte, PA) and helium as a carrier gas. The oven temperature was held at 150°C for 3 min, and then programmed from 150 to 210°C at 1°C per min. Fatty acids were identified by comparison of retention times with those of known standards (27).

Protein assay. Proteins were measured by the dye-binding method of Bradford (28) using bovine serum albumin as a standard.

Statistical analysis. Results presented in the tables and the figures are means ± SEM. Statistical significance of mean differences between dietary groups was investigated by analysis of variance (ANOVA) with the Sheffé multiple comparison method at $P \leq 0.05$ for lipid analyses and with the Fisher PLSD comparison method for phosphodiesterase activity, using the Statviews Program MacIntosh.

RESULTS

There was no significant difference in the body weight gains of rats fed the various diets over the three-week feeding period. Heart weights were not significantly different in rats fed sunflower oil or fish oil (in g, 1.36 ± 0.02 and 1.33 ± 0.06, respectively) but were found slightly but significantly increased ($P < 0.04$, 1.52 ± 0.06) in the coconut oil group. Also, there was no difference in the cytosolic to membrane protein ratios among the three dietary groups.

Feeding a diet supplemented with saturated, n-6 or n-3 fatty acids for a short-term period induced drastic changes in the fatty acid composition of both plasma (Table 3) and cardiac (Table 4) total phospholipids. Supplementing the diet with n-6 fatty acid rich sunflower oil increased the PUFA content of plasma phospholipids at the expense of monounsaturated fatty acids, although this diet contained the highest amount of oleic acid. The main fatty acid of the sunflower oil diet, 18:2n-6, was the highest in plasma phospholipid of this group. Supplementing the diet with n-3 rich fish oil significantly decreased the contents of 18:2n-6 and 20:4n-6 of plasma phospholipids and, in parallel, increased the contents of 20:5n-3, 22:5n-3 and 22:6n-3, resulting in a drastic fall in the n-6/n-3 fatty acid ratio as compared to those of the two other dietary groups. In addition, the total saturated fatty acid content of plasma phospholipids was significantly higher in the fish oil group than in the two other groups. The content of 18:2n-6 decreased only slightly in plasma phospholipids from rats fed coconut oil, despite the low amount of this fatty acid in the diet, while the 20:4n-6 concentration remained unchanged as compared with the n-6-enriched dietary group. Among the saturated fatty acids, 14:0 increased in the coconut oil group compared to the sunflower oil group while 16:0 and 18:0 were unaltered. The content of 16:0 was the highest in the fish oil group, and that of 18:0 was the lowest.

The total amount of fatty acids esterified into cardiac phospholipids was the same regardless of the diet (Table 4). Cardiac membranes maintained constant amounts of phospholipid saturated fatty acids, even in the sunflower oil group, which had the lowest level of these fatty acids in their plasma phospholipids. However, the PUFA distribution reflected the changes in the plasma fatty acid composition observed in each group. Thus, the n-6/n-3 fatty acid ratio was much lower in fish oil-fed rats than in sunflower oil and coconut oil fed rats. In the fish oil group, the content of 22:6n-3 in cardiac phospholipids was about sixfold higher than that of 20:5n-3, although 20:5n-3 was the main fatty acid of plasma phospholipids. The 22:5n-3 content of cardiac phospholipids was similar to that of 20:5n-3, whereas it was sixfold lower than the level of 20:5n-3 in plasma phospholipids. These data suggest that 20:5n-3 was either efficiently elongated or less

TABLE 3

Fatty Acid Composition of Serum Phospholipids from Rats Fed Various Diets[a]

Fatty acid	Sunflower oil	Fish oil	Coconut oil
14:0	0.19 ± 0.03[b]	0.39 ± 0.09[b,c]	0.55 ± 0.10[c]
16:DMA	0.21 ± 0.03[b]	0.28 ± 0.07[c]	0.16 ± 0.01[b]
16:0	20.60 ± 0.68[b]	27.90 ± 1.50[c]	20.50 ± 0.67[b]
16:1n-9	0.28 ± 0.02	0.30 ± 0.07	0.19 ± 0.02
16:1n-7	0.39 ± 0.04[b]	1.17 ± 0.20[c]	0.86 ± 0.10[b,c]
18:DMA	0.53 ± 0.15	0.64 ± 0.13	0.37 ± 0.11
18:0	20.80 ± 0.40[b]	18.40 ± 0.50[c]	21.80 ± 0.53[b]
18:1n-9	3.58 ± 0.10[b]	5.34 ± 0.24[c]	5.56 ± 0.13[c]
18:1n-7	2.07 ± 0.12[b]	2.99 ± 0.17[c]	3.10 ± 0.24[c]
18:2n-6	25.80 ± 0.40[b]	11.90 ± 0.57[c]	21.10 ± 0.37[d]
20:1n-9	0.15 ± 0.03[b]	0.28 ± 0.05[c]	0.09 ± 0.01[b]
20:2n-6	0.79 ± 0.06[b]	0.14 ± 0.02[c]	0.29 ± 0.01[d]
20:3n-6	0.49 ± 0.04[b]	0.62 ± 0.08[b]	1.19 ± 0.06[c]
20:4n-6	20.60 ± 0.65[b]	8.88 ± 0.35[c]	19.5 ± 0.34[b]
20:5n-3	n.d.	11.10 ± 0.73	n.d.
22:4n-6	0.78 ± 0.09	n.d.	0.49 ± 0.03
24:1n-9	0.10 ± 0.05	n.d.	0.09 ± 0.05
22:5n-6	1.40 ± 0.22[b]	0.09 ± 0.02[c]	1.70 ± 0.14[b]
22:5n-3	0.08 ± 0.01[b]	1.93 ± 0.23[c]	0.10 ± 0.01[b]
22:6n-3	1.15 ± 0.08[b]	7.51 ± 0.67[c]	1.90 ± 0.12[b]
UI	161	183	157
Saturated	41.63 ± 0.58[b]	46.70 ± 1.43[c]	42.95 ± 0.70[b]
Unsaturated	58.25 ± 0.56[b]	53.26 ± 1.43[c]	56.61 ± 0.71[b]
Monounsaturated	6.56 ± 0.24[b]	10.07 ± 0.49[c]	9.91 ± 0.41[c]
n-6	49.75 ± 0.67[b]	21.72 ± 0.61[c]	44.25 ± 0.78[d]
n-3	1.23 ± 0.08[b]	20.56 ± 1.51[c]	1.91 ± 0.14[b]
n-6/n-3	41.77 ± 2.70[b]	1.12 ± 0.12[c]	24.10 ± 1.67[d]

[a]Values are expressed as mol % of total fatty acids and are means ± SEM of nine different experiments. Values not bearing the same superscript letter are different at $P < 0.05$; UI, unsaturation index (sum of percentage of individual unsaturated fatty acid × number of double bonds); DMA, dimethylacetal; n.d., not determined.

TABLE 4

Fatty Acid Composition of Phospholipids from Rat Heart Membranes[a]

Fatty acid	Sunflower oil		Fish oil		Coconut oil	
14:0	n.d.		n.d.		2.91 ± 0.12	(0.5)
16:DMA	10.09 ± 0.31	(1.5)	8.89 ± 0.40	(1.6)	10.33 ± 0.66	(1.6)
16:0	53.77 ± 1.81[b]	(8.1)	58.03 ± 3.50[b]	(10.2)	70.03 ± 2.75[c]	(11.1)
16:1n-7	n.d.		4.17 ± 0.26	(0.7)	2.15 ± 0.21	(0.3)
18:DMA	5.72 ± 0.25[b]	(0.9)	3.99 ± 0.37[c]	(0.7)	3.66 ± 0.33[c]	(0.6)
18:0	164.34 ± 3.64	(24.8)	138.19 ± 8.76	(24.3)	139.15 ± 7.26	(22.1)
18:1n-9	19.88 ± 0.92[b]	(3.0)	15.26 ± 0.92[c]	(2.6)	25.39 ± 1.04[d]	(4.0)
18:1n-7	16.41 ± 0.40[b]	(2.5)	19.21 ± 1.23[b]	(3.4)	23.55 ± 0.89[c]	(3.7)
18:2n-6	176.66 ± 9.10[b]	(26.5)	90.88 ± 6.54[c]	(15.9)	146.78 ± 6.96[d]	(23.3)
20:4n-6	145.27 ± 7.35[b]	(21.9)	74.64 ± 3.52[c]	(13.1)	139.19 ± 5.80[b]	(22.1)
20:5n-3	n.d.		20.16 ± 1.17	(3.5)	n.d.	
22:4n-6	12.81 ± 0.82[b]	(1.9)	1.09 ± 0.12[c]	(0.2)	8.98 ± 0.57[d]	(1.4)
22:5n-6	24.85 ± 4.27[b]	(3.7)	1.88 ± 0.16[c]	(0.3)	22.75 ± 2.92[b]	(3.6)
22:5n-3	2.64 ± 0.21[b]	(0.4)	15.91 ± 0.76[c]	(2.8)	3.04 ± 0.18[b]	(0.5)
22:6n-3	26.48 ± 3.35[b]	(4.0)	116.51 ± 4.62[c]	(20.5)	32.38 ± 2.16[b]	(5.1)
Total	664.33 ± 21.53		568.44 ± 29.50		630.29 ± 25.16	
Unsaturated	446.21 ± 18.18[b]	(67.1)	372.22 ± 18.74[c]	(65.5)	418.20 ± 17.14[b,c]	(66.3)
Saturated	218.11 ± 4.42	(32.8)	196.22 ± 12.10	(34.5)	212.09 ± 9.85	(33.6)
n-6	364.94 ± 15.17[b]	(54.9)	168.12 ± 9.74[c]	(29.6)	317.69 ± 14.75[b]	(50.4)
n-3	29.13 ± 3.53[b]	(4.4)	152.58 ± 6.43[c]	(26.8)	35.42 ± 2.30[b]	(5.6)
n-6/n-3	13.30 ± 1.63[b]		1.10 ± 0.02[c]		9.14 ± 0.64[d]	

[a]Values are reported as μg fatty acid/mg protein and are means ± SEM (n = 6). Values in parentheses are % weight of total fatty acids. Values not bearing the same superscript letters are different at $P < 0.05$; DMA, dimethylacetal; n.d., not determined.

rapidly incorporated by cardiac membranes than other n-3 fatty acids. As previously observed in plasma phospholipids, the contents of 18:2n-6 and 20:4n-6 in cardiac phospholipids were markedly (−49%) depressed by fish oil feeding as compared to sunflower oil feeding. The level of 14:0, a fatty acid not detected in cardiac phospholipids of FO and SO groups, was increased in these phospholipids by coconut oil feeding. The 16:0 level was 20–30% higher in this group than in the two others, although the fatty acid was more abundant in plasma phospholipids from fish oil fed rats. Despite the low amount of available 18:2n-6 in the coconut oil-enriched diet, cardiac membrane efficiently incorporated this fatty acid into their phospholipids. The 18:2n-6 level was only slightly decreased (−17%) in the coconut oil group as compared to that of the sunflower oil group, whereas the 20:4n-6 level was not significantly different between both groups.

Variations observed in the nonesterified fatty acid content of rat heart membranes (Table 5) as a function of the different diets resembled those observed in the fatty acid content of cardiac phospholipids, but differences were found. Although the total fatty acid content of cardiac phospholipids was not affected by the diets, the nonesterified fatty acid content of heart membranes was 1.34- and 1.18-fold higher in the sunflower oil group and the fish oil group, respectively, than in the coconut oil group. The nonesterified PUFA content was the lowest in the coconut oil group, whereas cardiac phospholipid PUFA were most depressed in the fish oil group. This low amount of nonesterified PUFA in membrane fractions from coconut oil-fed rats as compared to sunflower oil-fed rats reflected the 51 and 27% decrease in the contents of 18:2n-6 and 20:4n-6, respectively, whereas only minor modifications of these two PUFA were observed in the cardiac phospholipid pool. In addition, fish oil feeding more drastically depressed the n-6/n-3 fatty acid ratio in the nonesterified fatty acid pool (71.5-fold SO *vs.* FO and 40-fold CO *vs.* FO) than in the cardiac phospholipid pool (12-fold SO *vs.* FO and 8.3-fold CO *vs.* FO).

The nonesterified fatty acid content of cytosolic heart fractions (Table 6) was far less affected by the diets than that of heart membrane. No difference was observed either in the total PUFA or in the total saturated fatty acid content of all groups. As previously observed in cardiac phospholipids, the nonesterified 18:2n-6 content was only slightly decreased (−29%), while the 20:4n-6 level did not change in the coconut oil group as compared to those of the sunflower oil group. Although less important than in the nonesterified fatty acid membrane pool, variations in the n-6/n-3 fatty acid ratios between the three dietary groups were markedly higher in the cytosolic compartment (31.3-fold SO *vs.* FO and 20.1-fold-CO *vs.* FO) than in cardiac phospholipids (12-fold SO *vs.* FO and 8.3-fold CO *vs.* FO).

The specific activity of cyclic nucleotide phosphodiesterase in the cytosolic and membrane fractions of rats fed the three diets is shown in Figure 1, A and B. Cyclic AMP phosphodiesterase specific activity was the lowest in both the soluble and particulate fractions of hearts from rats fed sunflower oil and the highest in the coconut oil group. In the cytosolic fraction, cyclic AMP phosphodiesterase activity of the CO group was 30% higher than that of the SO group ($P \leq 0.01$) and 15% higher than that of the FO group ($P \leq 0.05$). The difference observed between the SO and the FO groups was not significant. In the particulate fraction, the activity of the CO group was significantly higher (+18%, $P \leq 0.05$) than that of the SO group, but was not different from that of the FO group. In marked contrast, neither the basal level of cyclic GMP phosphodiesterase activity assayed in the presence of

TABLE 5

Nonesterified Fatty Acid Composition of Rat Heart Membranes[a]

Fatty acid	Sunflower oil	Fish oil	Coconut oil
14:0	0.302 ± 0.018 (2.6)	0.235 ± 0.021 (2.4)	0.319 ± 0.046 (3.8)
16:0	2.628 ± 0.110 (23.4)	2.277 ± 0.157 (23.0)	2.436 ± 0.224 (29.0)
16:1n-9	0.166 ± 0.022^{b} (1.4)	0.099 ± 0.011^{c} (1.0)	0.076 ± 0.009^{c} (0.9)
16:1n-7	0.133 ± 0.019 (1.1)	0.177 ± 0.020 (1.8)	0.144 ± 0.031 (1.7)
18:0	2.661 ± 0.103^{b} (23.7)	2.081 ± 0.085^{c} (21.0)	1.947 ± 0.085^{c} (23.2)
18:1n-9	1.559 ± 0.175 (13.9)	1.057 ± 0.126 (10.7)	1.159 ± 0.169 (13.8)
18:1n-7	0.222 ± 0.015 (2.0)	0.223 ± 0.007 (2.2)	0.282 ± 0.046 (3.4)
18:2n-6	2.431 ± 0.227^{b} (21.6)	1.124 ± 0.056^{c} (11.4)	1.244 ± 0.140^{c} (14.8)
20:4n-6	0.881 ± 0.061^{b} (7.8)	0.478 ± 0.032^{c} (4.8)	0.643 ± 0.049^{c} (7.7)
20:5n-3	n.d.	0.752 ± 0.082 (7.6)	n.d.
22:4n-6	0.091 ± 0.002 (0.8)	n.d.	0.050 ± 0.005 (0.6)
22:5n-6	0.060 ± 0.006 (0.5)	0.029 ± 0.004 (0.3)	0.044 ± 0.006 (0.5)
22:5n-3	n.d.	0.316 ± 0.041 (3.1)	n.d.
22:6n-3	0.057 ± 0.005^{b} (0.5)	1.066 ± 0.133^{c} (10.8)	0.060 ± 0.007^{b} (0.7)
Total	11.225 ± 0.499^{b}	9.892 ± 0.717^{b,c}	8.392 ± 0.724^{c}
Unsaturated	5.628 ± 0.383^{b} (50.1)	5.299 ± 0.477^{b,c} (53.5)	3.681 ± 0.430^{c} (43.9)
Saturated	5.597 ± 0.156 (49.9)	4.593 ± 0.252 (46.4)	4.702 ± 0.323 (56.0)
n-6	3.488 ± 0.272^{b} (31.1)	1.640 ± 0.085^{c} (16.6)	1.965 ± 0.194^{c} (23.4)
n-3	0.060 ± 0.003^{b} (0.5)	2.102 ± 0.265^{c} (21.2)	0.063 ± 0.008^{b} (0.7)
n-6/n-3	60.10 ± 7.80^{b}	0.84 ± 0.09^{c}	33.70 ± 5.60^{b}

[a]Values are reported as μg fatty acid/mg protein and are means ± SEM (n = 6). Values in parentheses are % weight of total fatty acids. Values not bearing the same superscript letters are different at $P < 0.05$; n.d., not determined.

TABLE 6

Nonesterified Fatty Acid Composition of Rat Heart Cytosol[a]

Fatty acid	Sunflower oil	Fish oil	Coconut oil
14:0	0.042 ± 0.006[b] (1.7)	0.051 ± 0.009[b,c] (2.0)	0.075 ± 0.010[c] (3.2)
16:0	0.488 ± 0.059 (19.4)	0.533 ± 0.058 (21.1)	0.514 ± 0.055 (21.8)
16:1n-9	0.037 ± 0.006 (1.5)	0.036 ± 0.005 (1.4)	0.030 ± 0.003 (1.2)
16:1n-7	0.032 ± 0.004[b] (1.3)	0.055 ± 0.007[c] (2.1)	0.042 ± 0.005[b] (1.8)
18:0	0.578 ± 0.076 (23.0)	0.552 ± 0.071 (21.9)	0.527 ± 0.087 (22.3)
18:1n-9	0.311 ± 0.046 (12.4)	0.279 ± 0.036 (11.1)	0.285 ± 0.037 (12.1)
18:1n-7	0.050 ± 0.008 (2.0)	0.064 ± 0.028 (2.5)	0.062 ± 0.007 (2.6)
18:2n-6	0.691 ± 0.061[b] (27.5)	0.399 ± 0.024[c] (15.8)	0.490 ± 0.052[c] (20.8)
18:3n-3	n.d.	n.d.	0.008 ± 0.001 (0.3)
20:4n-6	0.298 ± 0.047[b] (11.8)	0.188 ± 0.016[c] (7.4)	0.306 ± 0.046[b] (12.9)
20:5n-3	n.d.	0.189 ± 0.032 (7.5)	n.d.
22:5n-3	n.d.	0.051 ± 0.010 (2.0)	n.d.
22:6n-3	0.020 ± 0.009[b] (0.8)	0.160 ± 0.026[c] (6.4)	0.018 ± 0.03[b] (0.7)
Total	2.514 ± 0.282	2.512 ± 0.242	2.329 ± 0.243
Unsaturated	1.424 ± 0.052 (56.6)	1.404 ± 0.141 (55.9)	1.237 ± 0.147 (53.5)
Saturated	1.085 ± 0.130 (43.1)	1.107 ± 0.124 (44.1)	1.092 ± 0.149 (46.3)
n-6	0.989 ± 0.102[b] (39.3)	0.586 ± 0.036[c] (23.3)	0.801 ± 0.097[b,c] (33.9)
n-3	0.020 ± 0.009[b] (0.8)	0.383 ± 0.064[c] (15.2)	0.025 ± 0.005[b] (1.1)
n-6/n-3	59.52 ± 8.25[b]	1.90 ± 0.36[c]	38.26 ± 7.39[b]

[a]Values are reported as μg fatty acid/mg protein and are means ± SEM (n = 9). Values in parentheses are % weight of total fatty acids. Values not bearing the same superscript letters are different at $P < 0.05$; n.d., not determined.

ethyleneglyco-*bis*-(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (Fig. 1, A and B) nor the calmodulin-stimulated level was altered by the diets. In the membrane fraction from rat hearts where the cyclic GMP-stimulated isoform of phosphodiesterase is more preponderant, coconut oil feeding significantly increased (+20%, $P \leq 0.05$) the basal level of cyclic AMP phosphodiesterase activity as compared to the sunflower oil group while the cyclic GMP stimulated activity was not modified (Fig. 2). As a consequence, the stimulating effect of cyclic GMP upon cyclic AMP hydrolysis was significantly higher ($P \leq 0.01$) in the SO group (+272%) than in the CO group (+216%).

The *in vitro* influence of the main fatty acids of each diet upon the cytosolic PDE activity of hearts from rats fed a standard diet was also investigated. As shown in Table 7, polyunsaturated fatty acids proved to be more potent inhibitors than saturated fatty acids. Furthermore, the fatty acids affected cyclic AMP and cyclic GMP phosphodiesterase activities similarly.

DISCUSSION

The present results indicate that feeding rats an n-6 PUFA enriched diet for three weeks decreases cyclic AMP phosphodiesterase activity in both the cytosolic and membrane compartments. Feeding of n-3 PUFA does not significantly alter phosphodiesterase activity when compared with saturated fatty acid feeding. The fact that cyclic GMP hydrolysis remains unaffected by the various diets suggests that the calmodulin activated isoforms which hydrolyze the main part of cyclic GMP in heart cytosol are not very sensitive to dietary manipulations of the fatty acid composition of heart lipids.

None of the various diets used in the present study induced any significant difference in body weight of the animals at the end of the experiment, in accordance with previous results (4,10,14). However, Ruiz-Gutierrez *et al.* (29) reported that rats fed diets containing fish oil exhibited lower body weights than those fed corn, olive or soybean oil.

Marked changes in plasma phospholipid fatty acid composition were induced by feeding saturated, n-6 or n-3 fatty acids. Animals given diets enriched with saturated or n-3 fatty acids and supplemented with 2% of 18:2n-6-rich sunflower oil maintained a very low (undectable) level of 20:3n-9 (not shown). This indicated that the rats were not EFA deficient. The coconut oil-enriched diet, which contained 62% of fats as saturated fatty acids, did not increase the uptake of saturated fatty acids in plasma phospholipids. The saturated fatty acid level was higher in fish oil-fed animals which incorporated 16:0 more efficiently than rats from the two other groups. The monoenoic fatty acid proportion was significantly depressed in plasma phospholipids from rats fed the sunflower oil diet which contained the highest concentration of 18:1n-9, suggesting a competitive acylation between 18:1n-9 and 18:2n-6. In coconut oil-fed rats, the content of 18:2n-6 was significantly decreased compared to that of the SO group, while 20:4n-6 levels were similar in both groups. This result suggests that in the CO group the Δ6 desaturation/elongation process is efficient enough to maintain a 20:4n-6 concentration similar to that observed in sunflower oil-fed rats. In contrast, both 18:2n-6 and 20:4n-6 were replaced by n-3 fatty acids in the fish oil group, in accordance with previous reports (30).

The cardiac fatty acid composition markedly differed from that observed in plasma phospholipids. Although the total amount of fatty acids tended to be higher in the phospholipids of rats fed sunflower oil, this increase was only significant in the membrane pool of nonesterified fatty acids. No significant variations in the total saturated fatty acid levels were induced by the diet, whatever the considered cellular compartment. However, feeding a

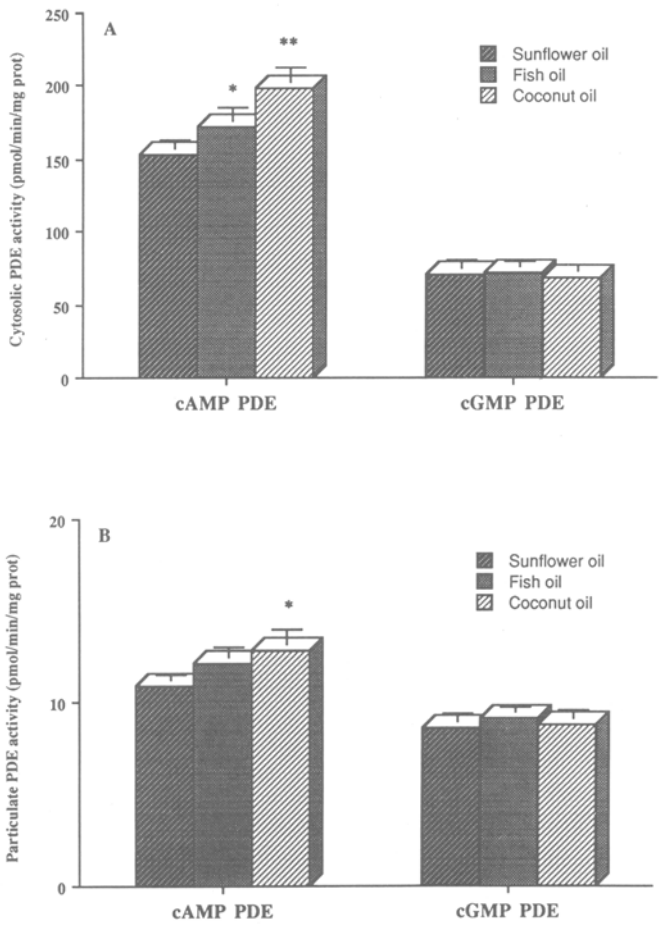

FIG. 1. Cardiac cyclic nucleotide phosphodiesterase activity from rats fed diets supplemented with sunflower oil, fish oil or coconut oil diets. Cytosolic (A) and particulate (B) fractions were prepared as described in Materials and Methods. Cyclic nucleotide phosphodiesterase activity was assayed with either 0.25 μM cyclic AMP or 0.25 μM cyclic GMP in the presence of 1 mM EGTA. * and **, Significantly different from the sunflower oil group at $P < 0.05$ and 0.01, respectively.

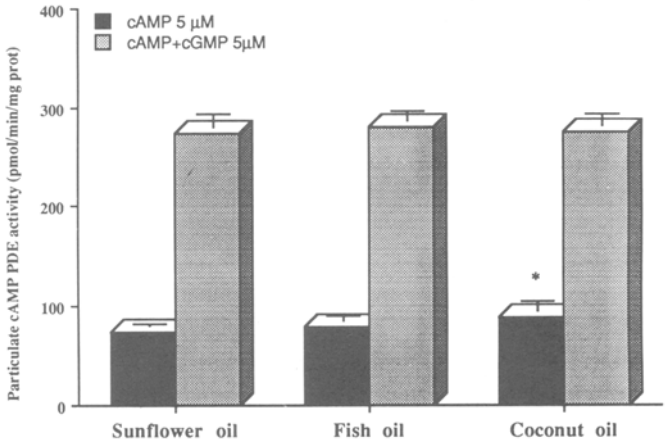

FIG. 2. Cyclic GMP-stimulated cyclic AMP hydrolysis in cardiac particulate fractions from rats fed diets supplemented with sunflower oil, fish oil or coconut oil diets. Cyclic AMP phosphodiesterase activity was assayed with 5 μM cyclic AMP, with or without 5 μM cyclic GMP. *, Significantly different from the sunflower oil group at $P < 0.05$. Percent stimulation of cyclic AMP hydrolysis by cyclic GMP was 272 ± 11, 255 ± 14 and 216 ± 13 for sunflower oil-, fish oil- and coconut oil-fed rats, respectively. Percent stimulation values of the coconut oil group were significantly different from values of the sunflower oil group at $P < 0.01$.

saturated fatty acid-enriched diet enhanced the level of monoenoic fatty acids in cardiac phospholipids, as previously reported by Awad and Chattopadhyay (9). The high saturated fatty acid content of the diet may stimulate Δ9 desaturase, hence increasing monoenoic fatty acid formation. Coconut oil feeding significantly decreased the total PUFA content only in the membrane nonesterified fatty acid pool, whereas normal levels of 18:2n-6 and 20:4n-6 were maintained in phospholipids, despite the low supply of 18:2n-6 in the diet. By contrast, the fish oil diet which provided the same amount of 18:2n-6 significantly decreased the n-6 fatty acid content of cardiac phospholipids when compared with the two other diets. This

TABLE 7

***In vitro* Influence of Fatty Acids on Cytosolic Cyclic Nucleotide Phosphodiesterase Activity from Heart of Rats Fed a Standard Diet[a]**

	IC_{50} or % variation at the indicated concentration in μM	
Fatty acids	Substrate: cAMP 0.25 μM	Substrate: cGMP 0.25 μM
14:0	301 (264-343)[b]	233 (211-258)
16:0	−17 at 117	−70 at 117
18:0	+9 at 130	−46 at 520
18:1n-9	148 (134-164)	54 (29-100)
18:2n-6	101 (59-173)	68 (58-80)
20:4n-6	94 (63-140)	63 (52-77)
22:6n-3	115 (105-125)	58 (31-109)

[a] Fatty acids were dissolved in ethanol and appropriately diluted with the enzyme assay buffer so that ethanol concentration did not exceed 1% vol/vol in the final assay medium. They were preincubated with the enzyme for 30 min at 37°C before the initiation of assay by substrate addition. Values are representative of two experiments performed in triplicate.

[b] Values in parentheses represent 95% confidence limits.

apparently results from a lower incorporation of 18:2n-6 due to the competitive replacement of n-6 by n-3 fatty acids. Furthermore, the low content of n-6 long chain fatty acids (22:4n-6 and 22:5n-6) in fish oil-fed animals may also result from the inhibition of Δ6 desaturase and elongase systems by n-3 PUFA (31,32).

Hearts from rats fed an n-3 fatty acid-rich diet selectively incorporated high amounts of n-3 PUFA, in agreement with results of others (10,14,15,33); high proportions of n-3 fatty acids were found in phospholipids as well as in the cytosolic and membrane nonesterified fatty acid pools. Specific acyltransferases selective toward some n-3 PUFA have been reported in heart tissue (34). Recently, Bouroudian *et al.* (35) have discovered the presence of an acyl-CoA synthetase with peculiar kinetic properties toward 22:6n-3, responsible for the inhibition of arachidonic acid incorporation into heart phospholipids. Whereas 20:5n-3 was the main n-3 fatty acid of plasma phospholipids, the concentration of 22:6n-3 was about fivefold higher than that of 20:5n-3 in heart phospholipids. Interestingly, Hagve and Sprecher (36) have shown that 20:5n-3 is taken up at a slower rate than other n-3 and n-6 fatty acids by isolated rat cardiac myocytes.

Several mechanisms might be involved in the lowering of cardiac cyclic AMP phosphodiesterase activity by sunflower oil feeding. Since n-6 polyunsaturated fatty acids are more potent inhibitors than saturated fatty acids (Table 7), the decrease in cyclic AMP phosphodiesterase activity observed after a three-week feeding period might result from a direct inhibitory effect by n-6 PUFA which accumulate more in the sunflower oil group than in the two other groups, especially in the membrane nonesterified fatty acid pool. However, *in vitro* addition of n-6 PUFA inhibited similarly cyclic AMP and cyclic GMP phosphodiesterase activities, whereas only cyclic AMP phosphodiesterase activity was affected by dietary manipulations. Thus, a direct interaction between nonesterified fatty acids and the phosphodiesterase isoenzymes may only partly explain the results obtained *in vivo* after a three-week feeding period.

Changes in the fatty acid composition of heart lipids also may influence phosphodiesterase activity through indirect and more complex mechanisms. The increase in nonesterified arachidonic acid content of the membrane pool caused by sunflower oil feeding may, in turn, increase prostanoid formation. However, no clear-cut relationship has yet been established between cellular eicosanoid content and phosphodiesterase activity. Several prostaglandins, as well as thromboxane B_2, were reported to have no direct effect on the activity of purified cyclic GMP-stimulated phosphodiesterase from calf liver *in vitro* (21). In platelets, prostaglandins are known to stimulate, through cyclic AMP-dependent phosphorylation, the activity of a cyclic AMP specific, cyclic GMP-inhibited isoform (37) which is found only in minor amount in rat heart tissue (38). Similarly, no direct influence of hydroperoxy fatty acids upon phosphodiesterase activity seems to exist in rat heart. An increased level of lipid peroxidation has been suggested to occur in fish oil-fed animals (39). However, PDE activity was not altered in the fish oil-fed group as compared with the coconut oil-fed group, suggesting that lipid peroxide levels do not directly influence cardiac phosphodiesterase activity, although some reports have indicated deleterious effect of hydroperoxides, such as H_2O_2 on platelet phosphodiesterase (40).

We have previously shown that compounds generated through phospholipid breakdown modulate cytosolic (41) as well as particulate (42) phosphodiesterase activities of rat heart. Synthetic diacylglycerols and phosphatidic acids were shown to stimulate cyclic AMP hydrolysis *in vitro*, dipalmitoyl phosphatidic acid being more efficient than egg yolk phosphatidate (42). Since the palmitic acid content of heart phospholipids was higher in the coconut oil-fed group than in the two other groups, coconut oil feeding may generate phosphatidate species containing higher levels of palmitic acid than PUFA feeding, thus increasing cyclic AMP phosphodiesterase activity.

Results of the present work showing that n-6 PUFA feeding and saturated fatty acid feeding are associated with decreased and increased cardiac cyclic AMP phosphodiesterase activity, respectively, might be physiologically relevant. Cyclic AMP phosphodiesterase inhibition is associated with increased intracellular cyclic AMP levels and increased cardiac contractility (18,19). Thus our results are in accordance with reports showing an increased sensitivity to catecholamines upon n-6 PUFA feeding (8) and with works reporting an impairment of cardiac functions associated with a relative n-6 fatty acid deficiency such as that induced by n-3 fatty acid feeding (15).

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The Effect of a Histidine-Excess Diet on Cholesterol Synthesis and Degradation in Rats

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Feeding a diet high in excess histidine (5% L-histidine) resulted in hypercholesterolemia and enlargement of the liver in rats. To clarify the mechanism of the hypercholesterolemia, cholesterol synthesis and degradation were followed. We found that hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in histidine-excess diet rats was significantly higher than in rats fed a basal diet. Incorporation of [^{3}H]water into cholesterol of liver slices from rats fed the histidine-excess diet was higher than incorporation into liver slices from rats fed the basal diet (expressed per liver per 100 g body weight). *In vivo* incorporation of [^{3}H]water into hepatic cholesterol was also higher, but the incorporation into cholesterol of the small intestine was lower in histidine-fed rats than in rats fed the basal diet (expressed per liver per 100 g body weight). Hepatic cholesterol 7α-hydroxylase activity was similar in both groups. The data suggest that the hypercholesterolemia caused by histidine-excess diet appears to be due to the stimulation of cholesterol synthesis in the liver. *Lipids 27*, 755–760 (1992).

In previous studies, growth retardation has been observed in rats fed diets enriched with a single amino acid (1). Several amino aicds were shown to affect cholesterol metabolism as well as growth. Dietary excess lysine (2,3), cystine (4,5) and histidine (2,6-11) can cause hypercholesterolemia in experimental animals. Solomon and Geison (6), Harvey *et al.* (7), Aoyama *et al.* (8-10) and Ohmura *et al.* (11) reported that dietary supplementation with excess-histidine leads to growth depression, hepatomegaly and increased plasma cholesterol levels in rats. Although histidine-fed rats did not ingest cholesterol, serum cholesterol significantly increased (6-11). Thus, the hypercholesterolemia induced by excess-histidine was not due to exogenous cholesterol. The distribution of serum high density, low density and very low density lipoprotein cholesterol in rats fed the histidine-excess diet was similar to that of rats fed the basal diet (11).

The mechanism of the increase in serum cholesterol has been investigated. Qureshi *et al.* (12) reported that the incorporation of [^{14}C]acetate or [^{14}C]mevalonate into cholesterol was significantly higher in the 5,000 × *g* liver supernatant fraction of histidine-supplemented chow-fed rats compared to controls. Solomon and Geison (13) also reported that a histidine-excess diet caused a 100% increase in the incorporation of [^{14}C]acetate or [^{14}C]octanoate into cholesterol of liver slices from weanling rats. These results suggested that cholesterol synthesis was stimulated by dietary excess histidine in the *in vitro* system. Cholesterol degradation has not been studied previously under histidine-excess dietary conditions.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

Because measurement of cholesterol synthesis with ^{14}C-labelled precursors may underestimate the actual rate of cholesterol formation (14-16), we used in the present study [^{3}H]water under *in vivo* and *in vitro* conditions to assess cholesterogenesis in animals fed the histidine-excess diet. It is well established that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase [EC.1.1.88] is the rate-limiting enzyme of cholesterogenesis (17-19) and that cholesterol 7α-hydroxylase [EC.1.14.13.7], the initial step in the conversion of cholesterol to bile acids, is the rate-limiting step in cholesterol degradation (20). We therefore examined liver HMG-CoA reductase and cholesterol 7α-hydroxylase activities to shed light on the regulation of cholesterol synthesis and degradation in rats fed a histidine-excess diet.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) were used. Rats were housed individually in screen-bottomed cages in an air-conditioned room kept at 22 ± 2°C on a 12-h light (0800-2000 h) dark (2000-0800 h) cycle. The rats were fed a basal diet (Table 1) for 3 to 5 days, allowing them to adapt to the shifted feeding pattern described below, and then were offered the experimental diets. The histidine-excess diet consisted of the basal diet with addition of 5% L-histidine (Ajinomoto Co., Inc., Tokyo, Japan) (Table 1). The addition of L-histidine was compensated for by adjusting the amount of carbohydrates. No dietary fibers were added to the experimental diets.

Changes in serum cholesterol (Experiment 1). Rats were fed the experimental diets for 0, 7 or 14 days. Diets were

TABLE 1

Composition of the Experimental Diets

Ingredient	Basal diet	His-excess diet[a]
	(%)	(%)
Casein[b]	25	25
L-Histidine[c]		5
Corn oil[d]	5	5
Vitamin mixture[e]	0.85	0.85
Choline chloride[f]	0.15	0.15
Mineral mixture[g]	3.5	3.5
Sucrose	21.8	20.2
Corn starch[g]	43.7	40.3
Retinyl palmitate[h]	0.27 mg/100 g of diet	
Cholecalciferol[h]	2.5 μg/100 g of diet	
dl-α-Tocopheryl acetate[h]	10 mg/100 g of diet	

[a]His, histidine.
[b]Katayama Chemical Industries Co., Ltd., Osaka, Japan.
[c]Ajinomoto Co., Inc., Tokyo, Japan.
[d]Nihon Syokuhin Kako Co., Ltd., Fuji, Japan.
[e]For details, see Aoyama, Y., and Ashida, K. (1972) *J. Nutr. 102*, 1025-1032.
[f]AIN-76^{TM} mineral mixture.
[g]Chuo Syokuryo Co., Ltd., Inazawa, Aichi, Japan.
[h]Eisai Co., Ltd., Tokyo, Japan.

offered from 1800 to 1000 h, and rats were sacrificed at 1000–1030 h by heart puncture.

HMG-CoA reductase activity (Experiment 2). Because food intake of rats fed the histidine-excess diet was significantly lower than that of rats fed the basal diet, a pair-fed basal group was used. Pair-fed rats were offered the same amount of a basal diet as consumed by the rats fed the histidine-excess diet. Diets were offered from 1800 to 1000 h, and the rats were sacrificed at 2300–2330 h by decapitation (21).

(i) Preparation of liver microsome. Aliquots of liver (1.5 g) were weighed and homogenized with 4 vol of cold buffer containing 0.3 M sucrose, 10 mM 2-mercaptoethanol, 10 mM sodium ethylenediaminetetraacetic acid (EDTA; pH 7.4) and 50 mM sodium fluoride using Potter-Elvehjem Teflon homogenizer (22). The resulting homogenate was centrifuged for 15 min at 12,000 × *g* at 4°C and the postmitochondrial supernatant was then centrifuged for 60 min at 105,000 × *g* at 4°C to sediment microsomes. The pellets were immediately stored at −80°C. Pellets could be stored for 10 days without loss of enzyme activity. Pellets were resuspended in 1 mL of preincubation medium before use.

(ii) Assay of microsomal HMG-CoA reductase activity. To assay the total activity of HMG-CoA reductase, the microsomes were preincubated at 37°C for 60 min with a preincubation mixture containing the following components in a volume of 45 μL: 20 mM imidazole chloride (pH 7.4), 5 mM dithiothreitol, 1.0–1.5 mg of microsomal protein and 10 units of alkaline phosphatase (*E. coli*) to convert "inactive" to "active" HMG-CoA reducase. For assay of native "active" HMG-CoA reductase activity, no alkaline phosphatase was added.

A solution (45 μL) containing 0.2 M potassium phosphate (pH 7.4), 20 mM NADPH, 20 mM sodium EDTA and 10 mM dithiothreitol was added to the preincubation mixture. The HMG-CoA reductase assay was then initiated by addition of DL[3-^{14}C]HMG-CoA (2,500 dpm/nmole)(52 mCi/mmol or 1.92 GBq/mmol)(Amersham Japan, Ltd., Tokyo, Japan) to a final concentration of 470 μM (final assay volume, 100 μL). After incubation for 30 min at 37°C, the [^{14}C]mevalonate formed was converted into lactone, isolated by thin-layer chromatography, using benzene/acetone (1:1, vol/vol) as the developing solvent, and counted using an internal standard of [^{3}H]mevalonate to correct for recovery. HMG-CoA reductase activity was expressed in nmoles of [^{14}C]mevalonate formed per hour per milligram of microsomal protein, nmoles per hour per gram of liver and nmoles per hour per liver per 100 gram of body weight.

In vivo *incorporation of [^{3}H]water into cholesterol (Experiment 3).* Rats were fed either a basal or a histidine-excess diet for 10 days. Diets were offered from 1000 to 1800 h. The rats were injected intraperitoneally with 250 μL of [^{3}H]water (1 mCi) (37 MBq) (Amersham Japan Ltd.). At 1 h after injection, the rats were sacrificed by heart puncture between 1400 and 1500 h. The liver and small intestine (0.5 cm below pylorus and 0.5 cm above cecum) were quickly removed. Intestinal contents were removed by rinsing with ice-cold saline. Both liver and small intestine were frozen in liquid nitrogen and stored at −20°C. An aliquot of the stored liver, small intestine and serum was saponified in 15% ethanolic potassium hydroxide for 60 min at 75°C. The hydrolysate was then extracted 4 times with petroleum hydrocarbon (b.p. 30–60°C). An aliquot of the petroleum hydrocarbon fraction was counted for radioactivity in the unsaponifiable fraction. Sterols in another of the petroleum hydrocarbon fraction were purified as digitonin-precipitable sterols by the method of Kelley and Tsai (23). The rate of synthesis was estimated by the formula of Turley *et al.* (24) based on serum ^{3}H-content. The rate of synthesis was expressed as nmoles of [^{3}H]water incorporated into digitonin-precipitable sterols per gram of tissue per h or nmoles per tissue per hour per 100 g body weight.

In vitro *incorporation of* [^{3}H]*water into hepatic cholesterol (Experiment 4).* The rats were fed either a basal or a histidine-excess diet for 10 days. Diets were offered from 1000 to 1800 h and rats were sacrificed between 1400 and 1500 h to prepare liver slices (0.5 mm thick) by hand. The liver slices weighing about 500 mg were incubated in 1.5 mL of Krebs Ringer bicarbonate buffer (pH 7.4) gassed with 95% O_2/5% CO_2 and containing 3.3 μCi (122 KBq) of [^{3}H]water (Amersham Japan Ltd.) and 16 μmoles of sodium acetate (25). At the end of the 2-h incubation with shaking at 37°C in an atmosphere of 95% O_2/5% CO_2, 2 mL of 15% ethanolic potassium hydroxide solution was added to the flask. The flasks' contents were saponified, extracted and purified as already described. The radioactivity of the nonsaponifiable fraction or digitonin-precipitable sterols was counted and expressed as μmoles of [^{3}H]water incorporated per gram of liver per hour or μmoles of ^{3}H per hour per liver per 100 gram body weight.

Assay of cholesterol 7α-hydroxylase (Experiment 5). Rats were fed the basal or the histidine-excess diet for 10 days. Pair-fed rats were offered the same amount of a basal diet as consumed by the rats fed the histidine-excess diet. Diets were offered from 1800 to 1000 h, and the rats were sacrificed between 2300 and 2330 h by decapitation.

(i) Preparation of liver microsome. Aliquots of liver (1.0 g) were weighted and then homogenized with a Potter-Elvehjem homogenizer with a Teflon pestle in 4 vol of ice-cold potassium chloride isotonic solution. The homogenate was centrifuged for 15 min at 12,000 × *g* at 4°C and the supernatant was then centrifuged for 60 min at 105,000 × *g* (4°C). The pellets were finally resuspended in 0.1 M potassium phosphate buffer (pH 7.4) (26,27).

(ii) Assay of microsomal cholesterol 7α-hydroxylase. The final incubation mixture (1 mL) contained 100 mM potassium phosphate buffer (pH 7.4), 20 mM cysteamine, 4 mM magnesium chloride, 5 mM NADPH and 1.0–1.5 mg of microsomal protein (26,27). The substrate [7(n)-^{3}H]cholesterol (5 μCi, 185 KBq) (7.45 Ci/mmol, 276 GBq/mmol) (Amersham Japan Ltd.) and 300 μM cholesterol were introduced into the incubation medium using a minimal volume of a concentrated solution in Tween-80 (26). Samples were incubated for 30 min in a shaking bath at 37°C. The enzymatic reaction was stopped by adding 3 vol of 20% (wt/vol) trichloroacetic acid solution. The tubes were centrifuged for 5 min at 3,000 × *g*. Then aliquots of the supernatants were transferred into appropriate tubes containing chloroform, and the content was extracted twice to remove unmetabolized [7(n)-^{3}H]cholesterol. The final water layer was counted for radioactivity.

Assay of serum and liver components. Lipids of the liver were extracted and purified according to the procedure of Folch *et al.* (28). Liver, serum and microsomal choles-

terol were measured enzymatically (29). Microsomal protein was measured by the method of Lowry *et al.* (30).

Statistical analysis. Data were analyzed by Student's *t*-test (31).

RESULTS

Changes in serum and liver cholesterol in rats fed either a basal or a histidine-excess diet. Food intake, body weight gain, serum cholesterol, liver weight and liver cholesterol of rats fed either a basal or a histidine-excess diet for 7 and 14 days are summarized in Table 2. Food intake and body weight gain were suppressed by the histidine-excess diet. Serum cholesterol and liver weight in rats fed the histidine-excess diet were significantly higher than those of rats fed the basal diet. Liver cholesterol content in rats fed the histidine-excess diet was lower when expressed as mg per gram liver, but higher when expressed as milligram per livers per 100 gram body weight than that in rats fed the basal diet.

Liver HMG-CoA reductase activity. Food intake, body weight gain, serum cholesterol, microsomal cholesterol and HMG-CoA reductase activity of rats fed a basal or a histidine-excess diet are summarized in Table 3. Food intake and body weight gain of rats fed a histidine-excess diet were lower than those of rats fed the basal diet *ad libitum* or those of pair-fed rats. Since pair-fed rats were fed the same amount of food eaten by rats fed the histidine-excess diet, body weight gain of pair-fed rats was similar to those of rats fed the histidine-excess diet. Serum cholesterol levels in pair-fed rats were higher than those in rats fed the basal diet *ad libitum*. Serum cholesterol levels in rats fed the histidine-excess diet were significantly higher than those in rats fed the basal diet *ad libitum* or those in pair-fed rats. Microsomal cholesterol levels were not affected by the histidine-excess diet. Total and "active" liver HMG-CoA reductase activities at midnight in rats fed the histidine-excess diet were higher than those in rats fed the basal diet *ad libitum* or those in pair-fed rats when expressed as milligram protein, gram liver or liver per 100 gram body weight. HMG-CoA reductase activity in pair-fed rats was similar to that in rats fed the basal diet *ad libitum*. The percentages of "active" activity in total activity were as follows: basal *ad libitum*, 25 ± 2; basal pair-fed, 26 ± 2; and histidine-excess *ad libitum*, 29 ± 2 (Experiment 2).

In vivo *and* in vitro *incorporation of [^{3}H]water into sterols.* The effects of dietary excess histidine on *in vivo* incorporation of [^{3}H]water into sterols are summarized in Table 4. *In vivo* incorporation of [^{3}H]water into liver digitonin-precipitable sterols, expressed as μmoles per hour per gram, in rats fed the histidine-excess diet was not significantly different from the incorporation in rats fed the basal diet. However, when expressed as μmoles per hour per liver per 100 gram body weight, the *in vivo* incorporation of [^{3}H]water into digitonin precipitable sterols was higher in rats fed the histidine-excess diet. Small intestine [^{3}H]digitonin-precipitable sterols, expressed as μmoles per hour per gram in rats fed the histidine-excess diet were similar to those in rats fed the basal diet. When expressed as μmoles per hour per tissue per 100 gram body weight, [^{3}H]digitonin-precipitable sterols in rats fed the histidine-excess diet was lower than that in rats fed the basal diet. There were no significant changes in serum [^{3}H]digitonin-precipitable sterols and serum ^{3}H-content between rats fed the basal and the histidine-excess diets.

In *in vitro* experiments (Table 5), ^{3}H-incorporation into the nonsaponifiable fraction was higher than that into the digitonin-precipitable sterols. ^{3}H-Incorporation into the nonsaponifiable fraction and digitonin-precipitable sterols in rats fed the histidine-excess diet was not significantly different from that in rats fed the basal diet when expressed as μmoles per hour per gram. When expressed as μmoles per hour per liver per 100 gram body weight, ^{3}H-incorporation into either fraction was signficantly higher in rats fed the histidine-excess diet than in rats fed the basal diet.

Cholesterol 7α-hydroxylase. Cholesterol 7α-hydroxylase activity of rats fed the histidine-excess diet (*ad libitum*) was similar to that of rats fed the basal diet (*ad libitum*). 7α-Hydroxylase activity of rats fed the histidine-excess diet was significantly higher than that of rats fed the basal diet (pair-fed) when expressed as nmoles per hour per liver per 100 kg body weight (Table 6).

DISCUSSION

Our observation that feeding a histidine-excess diet causes growth retardation, hepatomegaly and hypercholestrolemia is consistent with previous reports by Solomon and Geison (6), Aoyama *et al.* (8–10) and Ohmura *et al.* (11).

In the present study, 5% dietary L-histidine levels were used following the method of Solomon and Geison (6).

TABLE 2

Food Intake, Body Weight Gain, Serum Cholesterol and Liver Cholesterol in Rats Fed Either a Basal or a Histidine-Excess Diet (Experiment 1)

Diet	Days of feeding	Initial body wt (g)	Final body wt (g)	Food intake (g/7 or 14 days)	Body wt gain (g/7 or 14 days)	Serum cholesterol (mg/100 mL)	Liver wt (g/100 g body wt)	Liver cholesterol (mg/g)	Liver cholesterol (mg/liver/100 g body wt)
—	0	89.5 ± 2.6[a]	—	—	—	89.2 ± 4.6	4.02 ± 0.06	3.6 ± 0.1	14.4 ± 0.3
Basal	7	89.4 ± 1.7	117.4 ± 3.1	72 ± 3	28.0 ± 1.9	95.6 ± 3.6	3.74 ± 0.08	3.1 ± 0.1	11.7 ± 0.2
His-excess[b]	7	89.4 ± 2.0	103.0 ± 4.4[c]	54 ± 2[c]	13.6 ± 2.6[c]	170 ± 4[c]	6.18 ± 0.23[c]	2.5 ± 0.1[c]	15.2 ± 0.5[c]
Basal	14	89.8 ± 2.0	141.1 ± 0.9	147 ± 1	51.3 ± 1.2	99.3 ± 2.3	3.78 ± 0.07	3.1 ± 0.1	11.6 ± 0.2
His-excess	14	89.4 ± 1.5	128.0 ± 4.0[c]	129 ± 4[c]	38.6 ± 2.6[c]	173 ± 7[c]	6.11 ± 0.38[c]	2.5 ± 0.1[c]	14.9 ± 0.6[c]

[a]Means ± SEM for five rats.
[b]His, histidine.
[c]Significantly different from basal diet ($P < 0.05$).

TABLE 3

Food Intake, Body Weight Gain, Serum Cholesterol, Liver Microsomal Cholesterol and Liver HMG-CoA Reductase Activity in Rats Fed Either a Basal or a Histidine-Excess Diet for 10 Days (Experiment 2)

	Basal *ad libitum*	Basal pair-fed	His-excess[a] *ad libitum*
Initial body weight (g)	96.7 ± 1.6[b]	96.0 ± 0.8	99.1 ± 0.7
Final body weight (g)	145.8 ± 3.8	124.0 ± 1.0	126.9 ± 3.5
Food intake (g/10 days)	135 ± 2	96 ± 0[c]	96 ± 4[c]
Body weight gain (g/10 days)	49.1 ± 2.5	28.0 ± 0.4[c]	27.8 ± 3.0[d]
Serum cholesterol (mg/100 mL)	90.6 ± 1.5	101 ± 4	173 ± 4[c,d]
Liver weight (g/100 g body wt)	4.55 ± 0.03	4.49 ± 0.08	6.00 ± 0.15[c,d]
Liver microsomal cholesterol (μg/mg protein)	45.6 ± 1.8	44.8 ± 2.0	49.1 ± 2.6
Liver HMG-CoA reductase activity[e]			
Total activity			
(nmoles/h/mg protein)	2.00 ± 0.45	2.09 ± 0.16	5.17 ± 0.91[c,d]
(nmoles/h/g liver)	52.5 ± 6.1	62.0 ± 3.8	121 ± 17[c,d]
(nmoles/h/liver/100 g body wt)	239 ± 28	280 ± 20	721 ± 100[c,d]
"Active" activity			
(nmoles/h/mg protein)	0.50 ± 0.09	0.53 ± 0.03	1.49 ± 0.26[c,d]
(nmoles/h/g liver)	13.1 ± 2.1	15.9 ± 0.4	35.2 ± 5.1[c,d]
(nmoles/h/liver/100 g body wt)	59.7 ± 9.8	70.2 ± 4.3	208 ± 29[c,d]

[a]His, histidine.
[b]Means ± SEM for seven rats.
[c]Significantly different from basal *ad libitum* group ($P < 0.05$)
[d]Significantly different from basal pair-fed group ($P < 0.05$).
[e]The nmoles of mevalonate produced.

TABLE 4

***In vivo* ^{3}H Incorporation into Digitonin-Precipitable Sterols in Rats Fed Either a Basal or a Histidine-Excess Diet for 10 Days (Experiment 3)**

	Basal diet	His-excess diet[a]
Initial body weight (g)	89.5 ± 1.6[b]	90.1 ± 1.0
Final body weight (g)	115.4 ± 2.4	107.5 ± 1.9
Food intake (g/10 days)	96 ± 2	82 ± 3[c]
Body weight gain (g/10 days)	26.0 ± 1.0	17.4 ± 1.1[c]
Serum cholesterol (mg/100 mL)	89.9 ± 3.4	162.3 ± 5.6[c]
Liver weight (g/100 g body wt)	3.47 ± 0.07	5.14 ± 0.19[c]
In vivo ^{3}H-incorporation		
Liver [^{3}H]digitonin-precipitable sterols		
(μmoles/h/g liver)	0.24 ± 0.02	0.26 ± 0.03
(μmoles/h/liver/100 g body wt)	0.84 ± 0.06	1.47 ± 0.22[c]
Small intestine [^{3}H]digitonin-precipitable sterols		
(μmoles/h/g intestine)	0.43 ± 0.03	0.38 ± 0.01
(μmoles/h/intestine/100 g body wt)	1.00 ± 0.06	0.84 ± 0.04[c]
Serum [^{3}H]digitonin-precipitable sterols		
(μmoles/h/mL)	0.19 ± 0.04	0.16 ± 0.03
Serum ^{3}H content		
(dpm/mL × 10^{-7})	2.74 ± 0.07	2.82 ± 0.06

[a]His, histidine.
[b]Means ± SEM for ten rats.
[c]Significantly different from basal diet ($P < 0.05$).

In a preliminary study we had found that histidine levels higher than 3.5% in the diet induced hypercholesterolemia (Ohmura, E., Aoyama, Y. and Yoshida, A., unpublished data). The histidine levels used here should be considered pharmacological and/or toxicological and would not occur in a normal human diet. Thus, hypercholesterolemia caused by excess histidine should be seen as a model for endogenously induced hypercholesterolemia.

Dietschy and McGarry (14), and Stange and Dietschy (15) have demonstrated that ^{14}C-labeled substrates such as acetate or octanoate can be used for the estimation of sterols synthesis. Using ^{14}C-labeled substrate, the ability of the substrate to penetrate cell membranes, as well as intracellular dilution may have profound effects (16). Thus, methods based on ^{14}C-labeled substrates can underestimate (or sometimes overestimate) the true rate of cholesterol synthesis. Therefore, the use of [^{3}H]water seemed preferable for measuring the rates of cholesterogenesis. Rates of cholesterogenesis measured *in vivo* and *in vitro* (Tables 4 and 5) mostly reflect the activity of hepatic HMG-CoA reductase. Indeed, the activity of hepatic HMG-CoA reductase was higher in a histidine-

TABLE 5

***In vitro* ^{3}H Incorporation into Digitonin-Precipitable Sterols and Nonsaponifiable Fraction in Rats Fed Either a Basal or a Histidine-Excess Diet for 10 Days (Experiment 4)**

	Basal diet	His-excess diet[a]
Initial body weight (g)	85.8 ± 0.6[b]	85.8 ± 0.8
Final body weight (g)	109.3 ± 1.6	100.4 ± 0.9[c]
Food intake (g/10 days)	90 ± 2	78 ± 1[c]
Body weight gain (g/10 days)	23.5 ± 1.5	14.6 ± 1.0[c]
Serum cholesterol (mg/100 mL)	84.4 ± 10.1	191 ± 5[c]
Liver weight (g/100 g body wt)	3.95 ± 0.05	5.96 ± 0.11[c]
In vitro ^{3}H-incorporation using liver slices		
Digitonin-precipitable sterols		
(μmoles/h/g liver)	0.06 ± 0.01	0.06 ± 0.01
(μmoles/h/liver/100 g body wt)	0.25 ± 0.02	0.35 ± 0.02[c]
Nonsaponifiable fraction		
(μmoles/h/g liver)	0.11 ± 0.01	0.10 ± 0.01
(μmoles/h/liver/100 g body wt)	0.43 ± 0.03	0.59 ± 0.04[c]

[a]His, histidine.
[b]Means ± SEM for ten rats.
[c]Significantly different from basal diet ($P < 0.05$).

TABLE 6

Food Intake, Body Weight Gain, Liver Weight, Serum Cholesterol and Liver Cholesterol 7α-Hydroxylase Activity in Rats (Experiment 5)

	Basal *ad libitum*	Basal pair-fed	His-excess[a] *ad libitum*
Initial body weight (g)	124.2 ± 1.1[b]	123.1 ± 1.5	125.8 ± 1.1
Final body weight (g)	178.2 ± 2.0	155.5 ± 1.2	159.4 ± 2.4
Food intake (g/10 days)	141 ± 3	115 ± 0	113 ± 2[c]
Body weight gain (g/10 days)	54.0 ± 1.8	32.4 ± 1.2	33.6 ± 1.8[c]
Serum cholesterol (mg/100 mL)	90.2 ± 4.4	87.2 ± 4.0	171 ± 7[c,d]
Liver weight (g/100 g body wt)	4.41 ± 0.07	4.13 ± 0.03	5.85 ± 0.08[c,d]
Liver cholesterol 7α-hydroxylase			
(nmol/h/g protein	0.73 ± 0.05	0.60 ± 0.03[c]	0.61 ± 0.06
(nmol/h/g liver)	18.8 ± 1.7	15.5 ± 0.5	15.3 ± 1.7
(nmol/h/liver/100 g body wt)	82.9 ± 7.5	64.1 ± 2.4[c]	89.3 ± 9.6[d]

[a]His, histidine.
[b]Means ± SEM for eight rats.
[c]Significantly different from basal *ad libitum* group ($P < 0.05$).
[d]Significantly different from basal pair-fed group ($P < 0.05$).

excess group than in a basal group even though diets were offered from 1000 to 1800 h or 1800 to 1000 h (data not shown).

Food intake of rats fed the histidine-excess diet was lower than intake of rats fed the basal diet (Tables 2–6). To exclude the effect of reduced food intake due to excess histidine, pair-fed controls were used for following HMG-CoA reductase activity. Although food intake of pair-fed rats was restricted to 70% of that of rats that consumed the basal diet *ad libitum*, HMG-CoA reductase activity of pair-fed rats was not affected when compared with that of rats fed the basal diet *ad libitum* (Table 3). These results suggested that lower food intake in rats fed the histidine-excess diet had no effect on the activity of the enzyme. Thus, increased HMG-CoA activity in rats fed the histidine-excess diet appears to be due to excess histidine intake.

It is well known that HMG-CoA reductase activity is modulated by short-term and by long-term mechanisms (32–35). *In vivo*, short-term changes in enzyme activity can depend on the modulation of the enzyme by phosphorylation and dephosphorylation (32, 33), while long-term changes in the activity of the enzyme can depend on protein synthesis and/or degradation. In our study, long-term regulation of cholesterol synthesis may play a role in histidine-induced hypercholesterolemia because serum cholesterol in rats fed a histidine-excess diet was also increased by daytime feeding. Our results showed that the percentage of "active" activity in total activity was constant whether excess histidine was present or not (Table 3) suggesting long-term regulation rather than short-term regulation in the rats fed the histidine-excess diet. The total activity (active + inactive form) of HMG-CoA reductase is thought to reflect the protein level of HMG-CoA reductase in the liver (35). Our results (Table 3) showed that total HMG-CoA activity in rats fed the histidine-excess diet was significantly higher than that in rats fed the basal diet, suggesting that the content of HMG-CoA reductase protein may be higher in rats fed the histidine-excess diet as compared to that in rats fed the basal diet.

HMG-CoA reductase has a short half-life of about 2 h (36); so its level can easily be changed by dietary conditions (35) or circadian rhythm (37). Although the serum cholesterol level was high in rats fed the histidine-excess diet, the feedback system from serum cholesterol to hepatic HMG-CoA reductase might not be active.

We also examined that the possibility that histidine *per se* may be an *in vitro* activator of HMG-CoA reductase. L-Histidine was added to the assay mixture of HMG-CoA reductase. Microsomes obtained from rats fed the basal diet were used for the assay. The HMG-CoA reductase activities (nmoles per hour per milligram protein) were 1.35 for 0 mM histidine, 1.33 for 10 mM histidine and 1.66 for 50 mM histidine, suggesting that *in vitro* activation of HMG-CoA reductase by excess histidine may not be a factor. The possibility that histidine by itself is an activator of the enzyme can thus be excluded.

The activity of cholesterol 7α-hydroxylase, the regulatory enzyme of cholic acid synthesis, was unchanged by excess dietary histidine (Table 6). Thus, from both the increase in the activity of hepatic HMG-CoA reductase and the lack of an effect on cholesterol 7α-hydroxylase, the mechanism by which excess dietary histidine produces an increase in serum cholesterol appears to be the stimulation of cholesterol synthesis.

Total liver HMG-CoA reductase activity measured in rats fed during the light period and killed at midday was similar to results from rats fed during the dark period and killed at midnight. In rats fed during the light period and killed at midday, total liver HMG-CoA reductase activity in rats fed the histidine-excess diet was significantly higher than that in rats fed the basal diet when expressed as milligram protein or liver per 100 g body weight (not shown). Therefore, *in vivo* and *in vitro* incorporation of tritiated water was measured using rats fed during the light period and killed at midday (Experiments 3 and 4). Data obtained in the *in vivo* study (Table 4) suggest that cholesterol synthesis in the small intestine of rats fed a histidine-excess diet does not contribute to an increase in serum cholesterol.

[^{3}H]Digitonin-precipitable sterols in serum were measured to determine the export of newly synthesized cholesterol from tissues (Table 4). There was no difference in serum [^{3}H]digitonin-precipitable sterols between rats fed the basal diet or the histidine-excess diet. Work of Turley *et al.* (24) had indicated that nearly all [^{3}H]digitonin-precipitable sterols present in blood were derived from hepatic sources and that the high level of the liver [^{3}H]digitonin-precipitable sterols in rats fed the histidine-excess diet may not be due to decreased export of [^{3}H]digitonin-precipitable sterols from liver to serum. According to their results, the [^{3}H]digitonin-precipitable sterols in serum of rats fed the histidine-excess diet should be higher than that in rats fed the basal diet. However, this discrepancy may be due to the low amount of serum [^{3}H]digitonine-precipitable sterols exported within 60 min. More detailed investigations will be needed to shed light on the transport system for newly synthesized cholesterol.

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trans Fatty Acids. 5. Fatty Acid Composition of Lipids of the Brain and Other Organs in Suckling Piglets

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The effects of dietary *trans* fatty acids on the fatty acid composition of the brain in comparison with other organs were studied in 3-wk-old suckling piglets. In Experiment (Expt.) 1 the piglets were delivered from sows fed partially hydrogenated fish oil (PHFO) (28% *trans*), partially hydrogenated soybean oil (PHSBO) (36% *trans*) or lard (0% *trans*). In Expt. 2 the piglets were delivered from sows fed PHFO, hydrogenated fish oil (HFO) (19% *trans*) or coconut fat (CF) (0% *trans*) with two levels of dietary linoleic acid (1 and 2.7%) according to factorial design. In both experiments the mother's milk was the piglets' only food. The level of incorporation of *trans* fatty acids in the organs was dependent on the levels in the diets and independent of fat source (*i.e.*, PHSBO, PHFO or HFO). Incorporation of *trans* fatty acids into brain PE (phosphatidylethanolamine) was non-detectable in Expt. 1. In Expt. 2, small amounts (less than 0.5%) of 18:1 *trans* isomers were found in the brain, the level being slightly more on the lower level of dietary linoleic acid compared to the higher. In the other organs the percentage of 18:1 *trans* increased in the following order: heart PE, liver mitochondria PE, plasma lipids and subcutaneous adipose tissue. Small amounts of 20:1 *trans* were found in adipose tissue and plasma lipids. Other very long-chain fatty acids from PHFO or HFO (*i.e.*, 20:1 *cis* and 22:1 *cis* + *trans*) were found in all organ lipids except for brain PE. Dietary *trans* fatty acids increased the percentage of 22:5n-6 in brain PE. Except for the brain and the heart, dietary *trans* fatty acids reduced the percentage of saturated fatty acids and increased the percentage of monoenoic acids (including *trans*). The overall conclusion was that dietary *trans* fatty acids had no noticeable effect on the brain PE composition but slight to moderate effects on the fatty acid profile of other organs of suckling piglets.
Lipids 27, 761–769 (1992).

Human diets in Western society contain about 0.6-2 percent *trans* fatty acids (1,2) that mostly come from industrially processed fat with only small amounts being consumed in fats from ruminant animals. The fact that most of the *trans* fatty acids are of industrial rather than natural origin has been of concern and has led to a number of studies. *In vitro* studies have shown that *trans* fatty acids may inhibit the synthesis of longer chain essential n-6 and n-3 fatty acids from their lower homologues (3,4). These findings have led to speculation that *trans* fatty acids may interfere with the formation of eicosanoids (1,2,5,6) and may affect the biological properties of membranes (7).

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Abbreviations: CF, coconut fat; GLC, gas-liquid chromatography; HFO, hydrogenated fish oil; HPLC, high-performance liquid chromatography; LC, long-chain; PE, phosphatidylethanolamine; PHFO, partially hydrogenated fish oil; PHSBO, partially hydrogenated soybean oil; PUFA, polyunsaturated fatty acids; SFO, sunflower seed oil.

Because it has been shown that long-chain polyunsaturated fatty acids (LC-PUFA) are essential for nerve function (8,9), it is particularly important to ascertain whether consumption of *trans* fatty acids causes incorporation of *trans* fatty acids or alters the LC-PUFA profile in nerve tissue. In humans, brain growth occurs during late *prenatal* and early *postnatal* life (10), *i.e.*, the structural lipids of nerve tissues are formed in a relatively early stage. In a series of experiments comprising *prenatal* life (11) and the growth period to maturation (12), we have studied the effect of *trans* fatty acids with emphasis on nerve tissues. The observations include histology and functionality of nerve tissues (13) in addition to examining the content of *trans* fatty acids and fatty acid composition in general in the brain and several other organs (11,12). This final study concerned the effect of dietary *trans* fatty acids consumed from the mothers' milk during early *postnatal* life. The transfer of *trans* fatty acids from the mothers' diet to the milk has been reported (14). We have used pigs as models because of their physiological resemblance to humans (15,16), particularly with regard to brain growth (10) and accretion of LC-PUFA in nervous tissue (17). Further, we decided to focus on the very long-chain (*i.e.*, 20 and 22 carbon atoms) *trans* fatty acids found in partially hydrogenated fish oil (18); previous studies have mainly used partially hydrogenated vegetable oils that do not contain very long-chain *trans* fatty acids.

The present study consisted of two experiments. The first experiment compared diets with *trans* fatty acids from partially hydrogenated soybean oil and partially hydrogenated fish oil. The second experiment compared two fish oils differing in *trans* fatty acid contents when fed with two levels of dietary linoleic acid. Our analytical capacity did not allow us to analyze more than one phospholipid class. Since phosphatidylethanolamine (PE) has a high content of long-chain PUFA (19), which are assumed to be of particular importance for membrane structure and function, PE was chosen as the target lipid class. Lipid samples were obtained from the offspring after three weeks of suckling as the only nutrient supply. The brain was the principal organ of this investigation, but in order to compare the results from this study with those from previous ones, other tissues were analyzed as well.

EXPERIMENTAL PROCEDURES

General procedures. From 1 to 4 (depending on litter size) male and female piglets from each Norwegian Landrace sow were allowed to suckle and had no access to other feeds until they were three weeks old, when they were sacrificed by electrocution. Samples of 3 g each from heart, liver and adipose tissues, and 2.5 g from brain plus 1 mL of blood (right ventricle) plasma were immediately obtained for chemical analysis. Samples from individual piglets of each sex were pooled to two composite samples per sow (litter), *i.e.*, 8 (2 × 4) composite samples per diet. In Expt. 1 the piglets were bred from sows that from three weeks of age were fed diets based on cereals, solvent

extracted soybean meal, and 4 wt percent SFO (sunflower seed oil, 65% linoleic acid). To the respective diets were added 14 wt percent lard (control, diet 1), PHFO (partially hydrogenated capelin oil, m.p. 30–32°C, diet 2) or PHSBO (partially hydrogenated soybean oil, m.p. 40°C, diet 3). In Expt. 2, the piglets were bred from sows that through gestation were fed diets based on cereal grains and solvent extracted soybean meal to which was added 14 wt percent CF (coconut fat, diet 1.1 and 2.1), PHFO (diet 1.2 and 2.2) or HFO (hydrogenated fish oil, m.p. 50–52°C, diet 1.3 and 2.3) either without (diet 1.1, 1.2 and 1.3) or with (diet 2.1, 2.2 and 2.3) the addition of 3 wt percent SFO according to a 3 × 2 factorial design. Both experiments used four sows (litters). Details of the sows' diets and management have been presented earlier (13). The design of the experiments and the fatty acid composition of the sows' diets are shown in Table 1.

Chemical methods. Total lipids were extracted from the different organs by the method described by Folch *et al.* (20) except in the case of the brain, for which a slight modification of the Folch procedure was used (21). Membrane lipids of liver mitochondria were isolated as described by Høy and Hølmer (22). All lipids were stored at −25°C pending further analysis. Phosphatidylethanolamine (PE) of the liver mitochondria, heart and brain was isolated by high-performance liquid chromatography (HPLC). PE plasmalogen was not separated from PE since both coeluted in HPLC. The fatty acid composition reported for PE is therefore the combined composition of PE and PE plasmalogen. The fatty acid composition was analyzed by gas-liquid chromatography (GLC) using a capillary (CP Sil 88) column as described earlier (12). The contents of 16:1 *trans* fatty acids were determined only for the dietary fats used in Expt. 2 (Table 1). The 16:1 *trans* isomers of organ lipids could not be identified due to interference by other components on the chromatograms. Some organ lipids apparently contained 22:1 *trans*, but due to the low content, the *trans* and *cis* isomers could not be separated quantitatively.

Statistical methods. The basic principle of the statistical methods used was estimation of maximum likelihood. The program package used was BMDP, program P2V (23).

RESULTS

Brain. The fatty acid composition of brain PE is shown in Table 2. Although the percentages of some of the fatty acids in the offspring were significantly influenced by the dietary fats fed to the sows, the differences between diets were small. No *trans* fatty acids were incorporated into

TABLE 1

Fatty Acid Composition (%) of the Dietary Fats (total) Fed to Sows[a]

	Experiment 1[b]			Experiment 2					
	Added SFO (4%)			Added SFO (0%)			Added SFO (3%)		
	Lard	PHFO	PHSBO	CF	PHFO	HFO	CF	PHFO	HFO
Fatty acids	1	2	3	1.1	1.2	1.3	2.1	2.2	2.3
8:0				7.1			10.5		
10:0				6.1			5.8		
12:0				40.9	1.0	0.2	34.7	2.1	0.2
14:0				16.7	8.0	8.0	13.0	7.2	6.7
16:0	22.6	16.3	11.2	10.8	15.5	20.5	9.1	14.8	17.6
18:0	14.8	5.6	13.2	3.2	4.2	14.3	3.0	4.7	12.2
20:0	0.3	2.2	0.6		2.6	13.2		2.4	10.6
22:0		2.0			2.1	10.5		2.0	8.5
24:0					0.1	0.6		0.1	0.5
16:1 *cis*	1.5	1.1	0.6	0.2	2.9	0.5	0.2	2.7	0.6
16:1 *trans*[c]					4.8	1.9		3.9	1.9
18:1 *cis*	34.6	14.1	29.1	6.8	10.3	4.2	8.4	9.8	6.6
18:1 *trans*	0.3	6.8	24.3		7.7	3.1		5.8	2.9
20:1 *cis*	0.7	3.9	0.2		5.8	2.0		5.2	2.2
20:1 *trans*		6.0			9.5	5.9		7.9	5.3
22:1 *cis*		5.7			6.0	2.0		5.2	3.2
22:1 *trans*		5.3			6.7	4.4		5.5	3.6
24:1					0.5	0.6		0.4	0.5
18:2n-6	20.8	20.1	18.3	5:2	5.5	5.9	13.2	14.5	13.7
18:3n-3	1.8	2.2	1.9	2.2	1.6	1.4	1.8	1.1	1.3
Others	2.6	8.7	0.6	0.8	5.4	0.8	0.3	4.7	1.9
Sum PUFA[d]	22.6	22.3	20.2	7.4	7.1	7.3	15.0	15.6	15.0
Sum monoenoic	37.1	42.9	54.2	7.0	54.2	24.6	8.6	46.4	26.8
Sum saturated	37.7	26.1	25.0	84.8	33.5	67.3	76.1	33.3	56.3
Sum *trans*	0.3	18.1	24.3	0.0	28.7	15.3	0.0	23.1	13.7
P/S[e]	0.60	0.85	0.81	0.09	0.21	0.11	0.20	0.47	0.27

[a]Fed from 3 wk of age through pregnancy in Expt. 1, from gestation through pregnancy in Expt. 2.

[b]SFO, sunflower seed oil; PHFO, partially hydrogenated fish oil; PHSBO, partially hydrogenated soybean oil; CF, coconut fat.

[c]16:1 *trans* was not identified in Expt. 1.

[d]PUFA, polyunsaturated fatty acid.

[e]Relative amount of sum PUFA (P) to sum saturated fatty acids (S).

TABLE 2

Fatty Acid Composition (%) in Phosphatidylethanolamine of Total Lipids in Brain of 3-Wk-Old Suckling Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO (4%)					Added SFO (0%)			Added SFO (3%)				
	Lard	PHFO	PHSBO	Pooled std.	Significant experimental	CF	PHFO	HFO	CF	PHFO	HFO	Pooled std.	Significant experimental
Fatty acids	1	2	3	dev.	factors[b]	1.1	1.2	1.3	2.1	2.2	2.3	dev.	factors
14:0						0.1	0.2	0.1	0.3	0.2	0.2	0.2	
16:0	5.0	5.4	5.1	0.2		6.2	6.7	5.9	9.0	6.8	7.1	2.3	B
18:0	20.1	19.6	19.2	0.3	C	19.8	19.4	21.7	20.9	19.4	20.1	2.2	
16:1	0.8	0.9	1.0	0.1	C	0.7	0.9	0.8	0.3	0.6	0.5	0.4	B
18:1 *cis*	15.3	15.8	17.9	0.7	C	17.6	16.8	18.1	17.8	17.4	15.6	1.9	
18:1 *trans*	n.d.[c]	n.d.	n.d.			0.05	0.39	0.06	n.d.	0.18	0.05	0.09	A,B,C
20:1 *cis*	0.9	1.2	1.0	0.1	C	1.0	1.1	1.1	1.1	0.8	1.1	0.3	
20:3n-9	0.8	0.8	0.8	0.1		0.7	1.0	1.0	0.4	0.6	0.5	0.1	B,C
18:2n-6	1.3	1.8	2.1	0.2	C	1.4	2.0	1.0	1.4	1.6	1.4	0.9	
20:3n-6	0.9	0.8	0.9	0.1		0.9	1.0	1.0	0.8	0.9	0.8	0.2	B
20:4n-6	20.6	20.0	19.8	0.4	C	17.6	17.7	17.0	19.1	18.4	18.7	2.1	B
22:3n-6	0.5	0.7	0.6	0.1		0.7	0.7	1.0	0.5	0.6	0.7	0.3	B
22:4n-6	11.3	11.4	10.9	0.2		8.2	7.3	8.2	9.3	9.7	10.5	1.0	B
22:5n-6	2.6	4.7	3.6	0.3	C	1.5	1.5	1.3	1.9	2.8	2.7	0.4	A,B,C
22:5n-3	0.9	1.0	0.9	0.1		1.2	1.2	1.9	0.7	0.8	0.9	0.4	B,C
22:6n-3	18.5	15.8	15.5	0.6	C	18.2	18.4	15.8	13.8	16.2	16.6	2.1	A,B
Sum PUFAn-6	37.2	39.4	37.9			30.3	30.2	29.5	33.0	34.0	34.8		
Sum PUFAn-3	19.4	16.8	16.4			19.4	19.6	17.7	14.5	17.0	17.7		
Sum PUFA total	57.4	57.0	55.1			50.4	50.8	48.2	47.9	51.6	53.0		
Sum monoenoic	17.0	17.9	19.9			19.35	19.19	20.06	19.2	18.98	17.25		
Sum saturated	25.1	25.0	24.3			26.1	26.3	27.7	30.2	26.4	27.4		
Sum *trans*	0.0	0.0	0.0			0.05	0.39	0.06	0.00	0.18	0.05		

[a] From mothers fed these diets from 3 wk of age through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2). All abbreviations as in Table 1.

[b] Significance means ($P < 0.05$): A, Interactions of experimental fats and SFO-level; B, effect of SFO-level; C, effect of experimental fats. Average of eight replicates. Pooled std. dev., pooled standard deviation, (error mean square)$^{1/2}$.

[c] n.d., Not detectable.

brain PE in Expt. 1. In Expt. 2 low, but significant, amounts of *trans* 18:1 were detected when the sows were fed PHFO. The percentage of 18:1 *trans* was higher when PHFO was fed in combination with the lower level of linoleic acid compared with the higher level. In neither of the experiments was 20:1 *trans* found in the offspring. Percent 20:1 *cis* was slightly, but significantly, increased in offspring from sows fed PHFO in Expt. 1. This effect was not found in PHFO or in HFO in Expt. 2. Neither in Expt. 1 nor in Expt. 2 could 22:1 *cis* and *trans* be detected.

In Expt. 2, feeding hydrogenated fats to the sows increased the percentage of 20:3n-9 in their offspring. This effect was independent of the dietary level of linoleic acid. However, an increase in percent 20:3n-9 was also found as a consequence of low dietary levels of linoleic acid, independently of the content of hydrogenated fats in the diets.

In Expt. 1 the content of linoleic acid increased and the content of arachidonic acid decreased in the offspring when hydrogenated fats were fed to the sows. This was not observed in Expt. 2. In Expt. 2 hydrogenated fat increased the content of 22:5n-6. This was observed in Expt. 2 only when hydrogenated fat was fed in combination with the higher level of linoleic acid.

In Expt. 1, feeding PHFO and PHSBO to the sows reduced the content of 22:6n-3 in the offspring. In Expt. 2, PHFO and HFO increased 22:6n-3 in sows fed the higher level of EFA and had variable effects on sows on diets with restricted amounts of EFA.

Total content of PUFA was about 10% higher in Expt. 1 compared with Expt. 2. The fat sources fed to the sows had only minor effects on the total contents of saturated, monoenoic and polyunsaturated fatty acids in the offspring, but the higher dietary level of linoleic acid increased the contents of all n-6 fatty acids, except linoleic acid, and decreased the content of n-3 fatty acids.

Heart. The fatty acid composition of PE from the heart is shown in Table 3. In both experiments, feeding partially hydrogenated fat to the sows led to elevated levels of 18:1 *trans* in the offspring. However, lower but detectable levels of 18:1 *trans* were also found in offspring from the sows fed the control diets without partially hydrogenated fat. The content of 18:1 *trans* in the offspring was not influenced by the levels of linoleic acid in the diets of the sows in Expt. 2.

Feeding hydrogenated fats in combination with the lower level of linoleic acid to the sows in Expt. 2 increased the content of 20:3n-9 in the offspring. This effect of hydrogenated fats was not seen when fed together with the higher level of linoleic acid. Feeding partially hydrogenated fat to the sows in both experiments increased the content of linoleic acid and linolenic acid, and, except for the HFO in Expt. 2, decreased the content of arachidonic acid in the offspring. In both experiments, PHFO increased the content of 20:3n-6. PHFO and HFO in Expt. 2 increased the content of 20:5n-3.

The content of monoenoic fatty acids in the offspring reflected that of the fats fed to the sows in both

TABLE 3

Fatty Acid Composition (%) in Phosphatidylethanolamine of Total Lipids in Heart of 3-Wk-Old Suckling Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO (4%)			Pooled	Significant	Added SFO (0%)			Added SFO (3%)			Pooled	Significant
	Lard	PHFO	PHSBO	std.	experimental	CF	PHFO	HFO	CF	PHFO	HFO	std.	experimental
Fatty acids	1	2	3	dev.	factors[b]	1.1	1.2	1.3	2.1	2.2	2.3	dev.	factors
14:0						0.3	0.1	n.d.	0.6	0.1	0.1	0.3	C
16:0	5.2	6.1	4.9	1.0		5.0	6.7	5.1	8.8	5.8	4.1	2.8	A
18:0	21.1	15.2	12.9	1.0	C	22.2	17.8	20.7	20.3	19.7	21.2	2.4	
16:1	0.3	0.6	0.3	0.1	C	1.1	0.8	0.5	1.3	0.7	0.4	0.7	C
18:1 *cis*	8.4	10.3	13.6	1.1	C	8.8	12.2	9.4	10.3	10.2	7.4	3.1	
18:1 *trans*	0.8	1.9	3.8	0.8	C	0.1	2.7	0.6	0.5	2.4	1.0	0.8	C
20:1 *cis*	0.1	0.9	0.5	0.1	C	0.5	1.2	0.3	0.5	1.0	0.2	0.6	C
22:1 *cis* + *trans*	n.d.[c]	n.d.	n.d.			0.5	0.1	trace	0.2	0.4	0.1	0.5	
20:3n-9	0.2	0.1	0.1	0.1		0.7	1.2	1.1	0.4	0.4	0.5	0.2	A,B,C
18:2n-6	15.0	25.9	19.0	1.3	C	7.5	11.5	9.3	8.7	13.4	12.3	1.7	B,C
20:2n-6	0.4	0.3	0.3	0.1		0.2	0.3	0.2	0.2	0.2	0.2	0.2	
20:3n-6	0.9	1.1	0.8	0.1	C	1.1	1.5	1.2	0.7	1.2	0.9	0.2	B,C
20:4n-6	38.6	28.3	33.1	1.9	C	32.6	24.6	33.2	32.7	30.1	36.6	4.5	B,C
22:4n-6	1.6	1.4	1.6	0.3		0.9	0.5	0.9	1.3	1.1	1.3	0.3	B,C
22:5n-6	0.4	0.7	0.6	0.5		0.6	0.2	0.2	0.4	0.8	0.3	0.6	
18:3n-3	0.3	0.5	0.4	0.1	C	0.2	0.6	0.5	0.2	0.2	0.1	0.1	A,B,C
20:5n-3	1.3	1.4	1.5	0.2		4.9	6.9	5.8	0.9	1.1	2.1	1.4	B,C
22:5n-3	3.3	2.7	3.7	0.3	C	3.6	3.1	4.0	3.1	2.4	3.3	0.7	B,C
22:6n-3	2.8	2.4	2.8	0.5		3.4	2.9	3.0	2.6	2.5	2.6	0.6	B
Sum PUFAn-6	56.9	57.7	55.4			42.9	38.6	45.0	44.0	46.8	51.6		
Sum PUFAn-3	7.7	7.0	8.4			12.1	13.5	13.3	6.8	6.2	8.1		
Sum PUFA total	64.8	64.8	63.9			55.7	53.3	59.4	51.2	53.4	60.2		
Sum monoenoic	9.6	13.7	18.2			11.0	17.0	10.8	12.8	14.7	9.1		
Sum saturated	26.3	21.3	17.8			27.5	24.6	25.8	29.7	25.6	25.4		
Sum *trans*	0.8	1.9	3.8			0.1	2.7	0.6	0.5	2.4	1.0		

[a]From mothers fed these diets from 3 wk of age through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2). All abbreviations as in Table 1.

[b]Significance means ($P < 0.05$): A, Interactions of experimental fats and SFO-level; B, effect of SFO-level; C, effect of experimental fats. Average of eight replicates. Pooled std. dev., pooled standard deviation, (error mean square)$^{1/2}$.

[c]n.d., Not detectable.

experiments. However, only low levels of 22:1 *cis* plus *trans* were found and 20:1 *trans* was not detected.

In Expt. 1, feeding partially hydrogenated fat to the sows decreased the content of saturated fatty acids, increased the content of monoenoic acids but was without effect on the PUFA in the offspring. Similar effects were found for PHFO in Expt. 2, while HFO increased the content of PUFA but had only minor effects on the monoenoic acids. Increasing the level of linoleic acid in the sows' diets in Expt. 2 increased the content of the n-6 fatty acids and reduced the content of 20:3n-9 and n-3 fatty acids in the offspring.

Liver. The fatty acid composition of liver mitochondria PE from the piglets is shown in Table 4. In both experiments, feeding all types of partially hydrogenated fats to the sows increased the content of 18:1 *trans* in the offspring. Contrary to what was found for brain and heart, feeding of PHFO also led to incorporation of 20:1 *trans*. The level of linoleic acid in the sows' diets had no effect on the level of *trans* fatty acids in the offspring (Expt. 2).

The fatty acid profiles of the fats fed to the sows influenced the fatty acid composition of liver PE of the offspring.

In Expt. 1, PHSBO caused a marked increase in the percentage of 18:1 *cis* and a marked reduction in the percentage of 20:4n-6, 22:5n-3 and 22:6n-3. It is worth noting that despite a relatively high content of 20:1 *cis* and 20:1 *trans* and of 22:1 *cis* + *trans* in the diets of the sows fed PHFO and HFO, only very low to non-detectable levels of these fatty acids were found in the offspring. In total, feeding PHFO to the sows in both experiments increased the percentage of monoenoic acids and reduced the percentage of saturated acids. In Expt. 1, feeding PHFO increased and PHSBO decreased the percentage of PUFA in the offspring.

Increased levels of linoleic acid in the sows' diets in Expt. 2 reduced the percentage of 20:3n-9 and 20:5n-3 and increased the percentage of 18:2n-6, 20:4n-6 and 22:4n-6 in the offspring. Further, linoleic acid additions increased the percentage of PUFA n-6 and total PUFA and reduced the percentage of monounsaturated acids.

Blood. The fatty acid composition of total plasma lipids of venous blood is shown in Table 5. In both experiments, the percentage of 18:1 *trans* in the offspring increased significantly when the lactating sows were fed hydrogenated fats, but were not influenced by the dietary level of linoleic acid in Expt. 2. Feeding PHFO, and to a lesser degree HFO, to the sows gave appreciable levels of 20:1 *trans* in the offspring. In contrast to what was found for 18:1 *trans*, 20:1 *trans* was higher in piglets from sows fed the diet with the lower level of linoleic acid compared with the higher level. The percentages of 20:1 *cis*, 22:1 *cis* +

TABLE 4

Fatty Acid Composition (%) in Phosphatidylethanolamine of Liver Mitochondria of 3-Wk-Old Suckling Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO (4%)					Added SFO (0%)			Added SFO (3%)				
	Lard	PHFO	PHSBO	Pooled std. dev.	Significant experimental factors[b]	CF	PHFO	HFO	CF	PHFO	HFO	Pooled std. dev.	Significant experimental factors
Fatty acids	1	2	3			1.1	1.2	1.3	2.1	2.2	2.3		
14:0						0.2	0.3	0.1	0.1	0.2	0.2	0.1	A
16:0	7.7	7.5	8.5	1.2		10.1	8.4	8.4	8.3	6.2	7.7	1.5	B,C
18:0	35.9	23.8	27.9	5.4	C	34.2	25.4	26.9	28.7	25.6	27.8	4.4	C
16:1	0.5	0.8	1.0	0.3		1.1	1.4	1.0	0.7	0.5	0.9	0.5	B
18:1 *cis*	9.2	9.3	17.2	1.0	C	10.0	13.6	9.3	7.0	9.0	9.4	2.1	A,B,C
18:1 *trans*	0.1	6.0	7.6	1.3	C	0.6	4.2	1.1	0.1	4.0	1.2	0.9	C
20:1 *cis*	0.2	0.8	0.5	0.2	C	0.2	1.7	0.5	0.2	1.0	0.3	0.5	B,C
20:1 *trans*	n.d.[c]	0.3	n.d.		C	n.d.	0.3	trace	n.d.	0.1	trace	0.1	C
22:1 *cis* + *trans*	n.d.	n.d.	n.d.			0.1	0.2	0.2	n.d.	0.7	0.2	0.8	
20:3n-9	0.1	0.1	0.1	0.0		0.4	0.4	0.4	0.1	0.1	0.4	0.2	B
18:2n-6	9.2	12.9	9.0	1.7		6.7	7.3	7.0	8.4	9.0	8.7	1.5	B
20:2n-6	0.3	0.3	0.3	0.1		0.2	0.1	0.1	0.3	0.2	0.1	0.2	
20:3n-6	0.5	0.8	0.4	0.1	C	0.5	0.8	0.7	0.7	0.8	0.5	0.2	
20:4n-6	24.6	25.3	16.6	3.1	C	18.0	16.4	24.1	25.9	26.0	23.9	5.1	A,B
22:4n-6	1.0	1.0	0.6	0.2		0.7	0.4	0.9	1.9	0.8	1.3	0.7	B,C
22:5n-6	0.2	0.8	0.1	0.1	C	0.2	0.4	0.8	0.8	0.4	0.4	0.3	A
18:3n-3	0.2	0.1	trace	0.1	C	0.1	0.1	0.2	trace	0.1	0.2	0.1	C
20:5n-3	0.3	0.5	0.3	0.1		0.7	2.2	1.9	0.1	0.3	0.6	0.6	A,B,C
22:5n-3	2.6	2.7	1.1	0.4	C	2.4	2.6	4.0	3.0	2.7	3.1	1.0	C
22:6n-3	7.4	7.0	4.0	1.1	C	7.3	7.8	10.3	10.0	7.7	8.0	2.6	A
Sum PUFAn-6	35.8	41.1	27.0			26.3	25.4	33.6	38.0	37.2	34.9		
Sum PUFAn-3	10.5	10.3	5.4			10.5	12.7	16.4	13.1	10.8	11.9		
Sum PUFA total	46.4	51.5	32.5			37.2	38.5	50.4	51.2	48.1	47.2		
Sum monoenoic	10.0	17.2	26.3			12.0	21.4	12.1	8.0	15.3	12.0		
Sum saturated	43.6	31.3	36.4			44.5	34.1	35.4	37.1	32.0	35.7		
Sum *trans*	0.1	6.3	7.6			0.6	4.5	1.1	0.1	4.1	1.2		

[a] From mothers fed these diets from 3 wk of age through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2). All abbreviations as in Table 1.

[b] Significance means ($P < 0.05$): A, Interactions of experimental fats and SFO-level; B, effect of SFO-level; C, effect of experimental fats. Average of eight replicates. Pooled std. dev., pooled standard deviation, (error mean square)$^{1/2}$.

[c] n.d., Not detectable.

trans and 20:0 in the offspring increased when PHFO or HFO was fed to the sows in Expt. 2.

In Expt. 1 the percentage of linoleic acid increased, and in both experiments the percentage of arachidonic acid decreased in the offspring when hydrogenated fat was fed to the sows. Feeding CF led to a significant increase in the percentage of 12:0 and 14:0 in the offspring, especially when the sows were fed the diets with the lower level of linoleic acid.

The overall effect of feeding hydrogenated fat to the sows on the fatty acid composition in the offspring was a reduction in the percentage of saturated acids which was compensated for by an increase in the percentage of monounsaturated acids. Furthermore, the percentage of total n-6 PUFA was increased and the percentage of n-3 PUFA decreased when the sows were fed the diet with the higher level of linoleic acid compared with the lower level.

Adipose tissues. The fatty acid composition of the total lipids of subcutaneous adipose tissue of the piglets is shown in Table 6. In both experiments the percentage of 18:1 *trans* increased in the offspring when hydrogenated fats were fed to the sows. Furthermore, the percentage of 20:1 *trans* increased as a consequence of PHFO or HFO feeding, the percentage of 20:1 *trans* being higher when PHFO was fed together with the lower level of linoleic acid compared with the higher dietary level.

The percentage of 20:0, 20:1 *cis* and 22:1 *cis* + *trans* increased when PHFO or HFO was fed. Feeding hydrogenated fats to the sows had variable and inconsistent effects on the percentage of linoleic acid in the offspring. Thus, in Expt. 1, the percentage of linoleic acid increased when PHFO was fed, while the opposite occurred upon PHSBO-feeding. In Expt. 2 feeding PHFO and HFO increased the percentage of linoleic acid in the offspring when fed together with the higher dietary level of linoleic acid, while PHFO and HFO reduced the percentage of linoleic acid when fed together with the lower level of linoleic acid.

The percentage of arachidonic acid decreased in the offspring when PHFO or HFO was fed together with the lower dietary level of linoleic acid in Expt. 2 but not when fed together with the higher level. Feeding CF increased the percentage of 12:0 and 14:0.

As shown for the other organ lipids, feeding hydrogenated fats to the sows decreased the percentage of saturated fatty acids in the offspring and this was partly compensated by an increase in the percentage of monoenoic acids, *i.e.*, *cis* plus *trans* isomers.

TABLE 5

Fatty Acid Composition (%) of Total Plasma Lipids of Venous Blood from 3-Wk-Old Suckling Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO (4%)			Pooled std. dev.	Significant experimental factors[b]	Added SFO (0%)			Added SFO (3%)			Pooled std. dev.	Significant experimental factors
	Lard	PHFO	PHSBO			CF	PHFO	HFO	CF	PHFO	HFO		
Fatty acids	1	2	3			1.1	1.2	1.3	2.1	2.2	2.3		
12:0						2.5	n.d.	n.d.	1.7	n.d.	n.d.	0.5	A,C
14:0						4.8	2.0	2.6	3.5	1.7	2.3	0.7	B,C
16:0	22.0	18.6	14.0	1.0	C	22.6	18.1	21.0	21.8	16.7	20.5	0.9	B,C
18:0	12.2	8.4	8.1	0.5	C	11.9	8.7	10.4	12.6	9.7	10.3	1.5	A,B,C
20:0	n.d.[c]	n.d.		n.d.		trace	0.2	1.0	trace	0.2	0.4	0.3	A,C
16:1	2.1	2.6	1.3	0.2	C	4.1	4.4	4.4	2.4	2.6	2.9	0.6	B
18:1 *cis*	24.0	16.5	27.9	0.9	C	19.5	20.4	20.0	13.9	18.2	16.0	2.4	B,C
18:1 *trans*	n.d.	4.5	8.9	0.4	C	0.3	4.1	1.6	0.1	3.7	1.6	0.4	C
20:1 *cis*	0.3	1.4	0.7	0.2	C	0.2	1.9	1.8	0.2	1.5	1.4	0.5	C
20:1 *trans*	n.d.	0.5	n.d.	0.1	C	n.d.	1.5	0.2	n.d.	0.7	trace	0.7	A,C
22:1 *cis* + *trans*	n.d.	0.1	n.d.	0.0		0.1	0.7	0.2	0.1	0.3	trace	0.2	C
18:2n-6	27.9	36.5	29.8	1.1	C	19.3	18.2	18.3	27.5	28.5	28.2	1.9	B
20:2n-6	0.2	n.d.	0.1	0.1		0.2	0.3	0.2	0.2	0.2	0.2	0.1	A
20:3n-6	0.3	0.4	0.2	0.1	C	0.5	0.5	0.4	0.5	0.5	0.3	0.1	C
20:4n-6	6.0	5.2	5.0	0.2	C	5.3	3.7	5.1	7.5	5.5	6.0	0.8	C
22:4n-6	0.1	0.1	0.1	0.1		0.1	n.d.	0.1	0.3	0.1	0.1	0.1	A,C
18:3n-3	1.1	1.4	1.2	0.3		1.4	1.5	1.2	0.8	0.8	1.0	0.4	B
20:5n-3	0.3	0.3	0.2	0.1		1.2	1.1	1.1	0.4	0.4	0.4	0.2	
22:5n-3	0.8	0.7	0.6	0.2		1.1	1.0	1.3	1.1	0.8	1.0	0.2	A,C
22:6n-3	1.6	1.1	1.0	0.1	C	2.0	2.0	1.9	2.1	1.5	1.6	0.3	C
Sum PUFAn-6	34.5	42.2	35.2			25.4	22.7	24.1	36.0	34.8	38.8		
Sum PUFAn-3	3.8	3.5	3.0			5.6	5.6	5.5	4.4	3.5	4.0		
Sum PUFA total	38.3	45.7	38.2			31.0	28.3	29.6	40.4	38.3	38.8		
Sum monoenoic	26.4	25.6	38.8			24.2	33.0	28.2	16.7	27.0	21.9		
Sum saturated	34.2	27.0	22.1			41.8	29.0	35.0	39.6	28.3	33.5		
Sum *trans*	0.0	5.0	8.9			0.3	5.6	1.8	0.1	4.4	1.6		

[a]From mothers fed these diets from 3 wk of age through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2). All abbreviations as in Table 1.

[b]Significance means ($P < 0.05$): A, Interactions of experimental fats and SFO-level; B, effect of SFO-level; C, effect of experimental fats. Average of eight replicates. Pooled std. dev., pooled standard deviation, (error mean square)$^{1/2}$.

[c]n.d., Not detectable.

Further additions of linoleic acid to the sows' diets increased the percentage of n-6 PUFA but had little effect on the percentage of n-3 PUFA in the offspring.

DISCUSSION

Nervous tissue contains large quantities of long-chain polyunsaturated, essential n-6 and n-3 fatty acids (LC-PUFA) which play important roles in nerve structure and function (24). It is therefore anticipated that nutritional deficiencies and imbalances and metabolic disorders that affect fatty acid metabolism may have a particularly detrimental effect on nervous tissue. Accretion of nervous tissue occurs over a relatively brief period of time during late gestation and early *postnatal* life (10,25). Since *in vitro* (3,4,26) and *in vivo* (27–29) studies have indicated that *trans* fatty acids may interfere with the metabolism of essential fatty acids, the content of *trans* fatty acids in the maternal diet during pregnancy and nursing is of concern for the development of nervous tissue in the offspring. Our previous studies showed no effect of *trans* fatty acids in the maternal diet on nervous tissue histology and function in 3-wk-old suckling piglets (13). However, we did find that *trans* fatty acids in the diets of the sows were transferred into the milk but had only marginal effects on the overall fatty acid composition of the milk (14).

The present study focused on the effect of the content of *trans* fatty acids in the maternal diet (and consequently in the milk) on the fatty acid composition of the offspring after nursing for three weeks after birth. In addition, comparisons were made in Expt. 1 between a hydrogenated fat of vegetable origin (PHSBO) containing *trans* fatty acids with 16 and 18 carbons and a hydrogenated fat of fish origin (PHFO) which in addition to C_{16} and C_{18} *trans* fatty acids also contained *trans* fatty acids with 20 and 22 carbons. Since dietary levels of linoleic acid have been claimed to influence the effect of *trans* fatty acids (1,30), different levels of *trans* fatty acids in hydrogenated fish oils were studied at two levels of dietary linoleic acid in Expt. 2.

The general outcome of the study is consistent with the results of our previous investigations and with mature pigs being fed diets with *trans* fatty acids for a six-month period (12). Thus, *trans* fatty acids present in the milk were absorbed by the young piglets and deposited in various tissues. These results are also consistent with previous findings in suckling rats (31). In agreement with our earlier results with new born (11) and mature (12) pigs, the deposition of *trans* fatty acids varied greatly between different organs, being highest in adipose tissue and lowest in brain. Thus, despite the fact that appreciable levels of *trans* fatty acids were present in the milk (14),

TABLE 6

Fatty Acid Composition (%) of Subcutaneous Adipose Tissue of 3-Wk-Old Suckling Piglets[a]

	Experiment 1					Experiment 2							
	Added SFO (4%)			Pooled std. dev.	Significant experimental factors[b]	Added SFO (0%)			Added SFO (3%)			Pooled std. dev.	Significant experimental factors
	Lard	PHFO	PHSBO			CF	PHFO	HFO	CF	PHFO	HFO		
Fatty acids	1	2	3			1.1	1.2	1.3	2.1	2.2	2.3		
12:0						3.4	0.1	0.1	2.9	0.1	trace	0.5	C
14:0						11.0	4.8	5.2	10.2	3.9	4.3	0.9	B,C
16:0	21.4	20.8	14.1	0.5	C	26.2	20.1	25.5	24.6	18.1	23.3	1.3	B,C
18:0	6.3	4.2	5.3	0.5	C	5.1	3.7	5.8	5.1	4.2	5.5	0.7	C
20:0	n.d.[c]	n.d.	n.d.			0.1	0.4	0.9	0.1	0.4	1.0	0.1	A,C
16:1	3.8	7.5	2.7	0.3	C	8.8	8.2	8.6	6.9	5.8	5.0	0.5	A,B,C
18:1 *cis*	44.4	26.9	42.9	1.5	C	31.8	30.1	34.9	30.3	29.3	29.0	3.1	
18:1 *trans*	n.d.	5.1	11.0	1.0	C	0.1	6.3	2.4	0.1	6.1	2.2	0.7	C
20:1 *cis*	0.7	4.0	1.2	0.4	C	0.4	4.8	3.1	0.4	4.7	2.4	0.8	C
20:1 *trans*	n.d.	3.8	0.2	0.3	C	n.d.	2.7	0.8	n.d.	2.1	0.9	0.3	A,C
22:1 *cis* + *trans*	0.1	1.0	n.d.	0.1	C	trace	1.1	0.1	trace	1.0	0.3	0.2	A,C
20:3n-9	0.6	1.0	0.8	0.2		0.1	0.1	0.1	0.2	0.2	0.2	0.1	B
18:2n-6	20.0	22.5	18.4	0.5	C	6.9	5.8	6.3	14.5	15.6	17.8	0.8	A,B,C
20:2n-6	0.2	0.2	0.1	0.1	C	0.2	0.4	0.1	0.3	0.5	0.3	0.1	C
20:4n-6	0.6	0.8	0.5	0.3		0.9	0.3	0.4	0.8	0.5	0.9	0.3	A,B
18:3n-3	1.7	1.8	1.5	0.1		1.4	1.3	1.6	1.2	1.1	1.3	0.2	B,C
22:5n-3	0.2	trace	trace		C	0.4	0.2	0.1	0.3	0.1	0.3	0.1	A,C
22:6n-3	0.1	0.1	0.1	0.1		0.3	0.1	0.1	0.2	0.1	0.2	0.1	C
Sum PUFAn-6	20.8	23.5	19.0			8.0	6.5	6.8	15.6	16.6	19.0		
Sum PUFAn-3	2.0	1.9	1.6			2.1	1.6	1.8	1.7	1.3	1.8		
Sum PUFA total	23.4	26.4	21.4			10.2	8.2	8.7	17.5	18.1	21.0		
Sum monoenoic	49.0	48.3	58.0			41.1	53.2	49.9	37.7	49.0	39.8		
Sum saturated	27.7	25.0	19.4			45.8	29.1	37.5	42.9	26.7	34.1		
Sum *trans*	0.0	8.9	11.2			0.1	9.0	3.2	0.1	8.2	3.1		

[a]From mothers fed these diets from 3 wk of age through pregnancy (Expt. 1) and from gestation through pregnancy (Expt. 2). All abbreviations as in Table 1.

[b]Significance means ($P < 0.05$): A, Interactions of experimental fats and SFO-level; B, effect of SFO-level; C, effect of experimental fats. Average of eight replicates. Pooled std. dev., pooled standard deviation, (error mean square)$^{1/2}$.

[c]n.d., Not detectable.

trans fatty acids were not detected in the brains of the offspring in Expt. 1, and only low levels of 18:1 *trans* and no 20:1 *trans* were found in Expt. 2. These results are in entire agreement with our previous results from mature and newborn pigs (11,12). In experiments with rats, Cook (32) found that small amounts (0.2%) of elaidic acid (18:1 *trans*) injected into the stomach of young rats were recovered in the brain. It therefore appears that although *trans* fatty acids may pass the blood-brain barrier in suckling animals, the amount actually being deposited in the brain is indeed very limited.

Table 7 shows the discrimination factors (*i.e.*, the relation of the percentage of *trans* isomers of the total monoene contents of the organ lipids to that of the respective dietary lipids) in the different organs of the 3-wk-old piglets compared to those of mature (12) and *neonatal* (11) pigs. In order to relate the findings in the 3-wk-old piglets to maternal nutrition, the calculation is based both on the milk and on the sows' diets. The relative values between the different organs were similar in 3-wk-old and mature pigs, but relatively more *trans* fatty acids from the diet (*i.e.*, milk) were incorporated into the organs of the 3-wk-old pigs compared to the mature pigs.

Indeed, the percentage of the *trans* fatty acids in the mothers' diet incorporated into the suckling piglets was similar to that found in the mothers themselves (12). It is possible that the relatively high incorporation of *trans* fatty acids in the 3-wk-old pigs was due to a low *de novo* fatty acid synthesis in these animals consuming milk in which a very high portion of the total energy came from fat. Except for liver mitochondria in which relatively more *trans* fatty acids were incorporated from the PHFO than from PHSBO, the discrimination factors were similar for the two fat sources in common with that found for mature pigs. With the exception of the brain, in which the incorporation of 18:1 *trans* was significantly increased on the lower dietary levels of linoleic acid compared with the higher levels, dietary levels of linoleic acid did not affect the incorporation of *trans* fatty acids. This result is contradictory to those of Hill *et al.* (33) who found a significant effect from dietary levels of linoleic acid on *trans* fatty acid incorporation. The reason for this discrepancy is not immediately evident.

To what extent the levels of *trans* fatty acids in the organs continue to increase over a life span is an intriguing question. The newborn and 3-wk-old pigs were offspring of the mature pigs used in this study (*i.e.*, Expt. 1), and all pigs were subjected to the same dietary regimen. The data therefore lend themselves to showing age effects on the levels of *trans* fatty acids in various organs. Figure 1 shows the percentage of *trans* fatty acids in PE of heart, liver mitochondria and adipose tissue of newborn, 3-wk-old and mature pigs when fed PHFO and PHSBO in Expt. 1. The figure shows that in these organs there was a sharp

TABLE 7

Ratio of Percentage *trans* Isomers of the Total Monoene Content of Organ Lipids to that of the Respective Dietary Fats in 3-Wk-Old Suckling Piglets, Newborn Piglets (11) and Their Adult Mothers (12) in Experiment 1[a]

	18:1 *trans*		20:1 *trans*
	PHFO	PHSBO	PHFO
Brain			
Adult	0	0	0
Neonatal	0	0	0
Postnatal/diet	0	0	0
Postnatal/milk	0	0	0
Heart			
Adult	0.45	0.32	0.15
Neonatal	0.17	0.13	0
Postnatal/diet	0.48	0.48	0
Postnatal/milk	0.70	0.69	0
Liver			
Adult	0.94	0.76	0.41
Neonatal	0.37	0.31	0
Postnatal/diet	1.21	0.67	0.45
Postnatal/milk	1.75	0.97	0.69
Blood			
Adult	0.63	0.51	0
Neonatal	0.20	0.14	0
Postnatal/diet	0.66	0.53	0.43
Postnatal/milk	0.96	0.78	0.66
Adipose			
Adult	0.64	0.58	0.87
Neonatal	0	0.01	0
Postnatal/diet	0.49	0.45	0.80
Postnatal/milk	0.71	0.64	1.23

[a]Fed the diets from 3 wk of age through gestation. For the suckling piglets (*i.e., postnatal*) discrimination factors based on the respective milk lipids (14) also were calculated. All abbreviations as in Table 1.

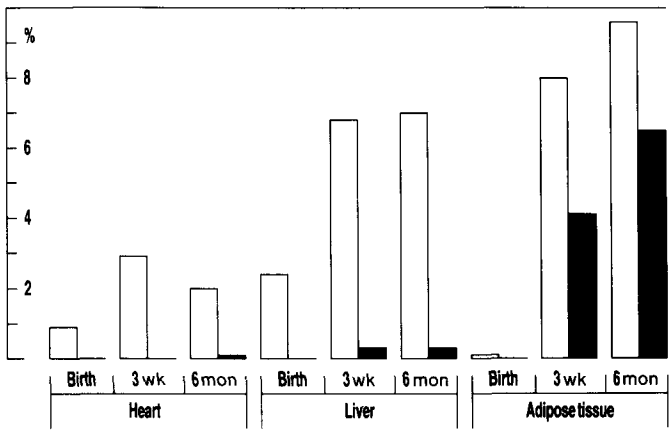

FIG. 1. Average percentage of 18:1 *trans* (white bars; animals fed PHFO and PHSBO) and 20:1 *trans* (black bars; animal fed PHFO) in organs of newborn, 3-wk-old, and mature pigs.

increase in the percentage of *trans* fatty acids during the first three weeks of life and that no consistent changes occurred thereafter. It thus appears that the content of *trans* fatty acids reaches a steady level early in life and is dependent on the dietary level of *trans* fatty acids.

Previous *in vitro* studies have shown that *trans* fatty acids may interfere with the metabolism of the essential n-6 and n-3 fatty acids through inhibition of the Δ5 (4,26) and Δ6 (3,4,26,30) desaturases. If such effects were of significant magnitude, they could influence fatty acid composition of the brain and consequently brain function. In the present study the effects of *trans* fatty acids on brain fatty acid composition were negligible although a slight increase in 18:2n-6 and 22:5n-6, and a small and inconsistent decrease in 20:4n-6 and 22:6n-3 were seen. These findings are in agreement with our previous results on newborn (11) and mature (12) pigs and with those of Lawson *et al.* (27) on rats being fed *trans* fatty acid for two generations. They are also in agreement with the findings of Sanders *et al.* (34) who found a reduction in brain 22:6n-3 and an increase in brain 22:5n-6 when fed *trans* fatty acid containing margarine to rats.

The effect of *trans* fatty acids on the fatty acid composition (*i.e.,* the levels of n-6 and n-3 fatty acids) in the other organs was similar in nature but of somewhat greater magnitude than that found in the brain, and this is in agreement with our previous results with newborn (11) and mature pigs (12).

This paper concludes a series of experiments with the aim of studying the effects of dietary *trans* fatty acids, with emphasis on nervous tissue. Since knowledge of the effect of long-chain *trans* fatty acids is meager, comparisons were made between partially hydrogenated soybean oil and partially hydrogenated fish oil. Pigs were used as model animals due to their resemblance to humans with regard to lipid metabolism and nerve tissue accretion (10,17). The study comprised the intrauterine period, early *postnatal* life (suckling period) and the growth period through the first pregnancy.

The pigs were fed diets that resembled human diets in Western society with regard to content of fat (about 40 cal%) and linoleic acid (2 to 9 cal%) and had a P/S (polyunsaturated fatty acids/saturated fatty acids) ratio from about 0.1 to 0.8, but contained 2 to 4 times more *trans* fatty acids (*i.e.,* 6 to 12 cal% *vs.* 3–4 cal%) (1). The combined results allow us to draw the following conclusions. *trans* Fatty acids were absorbed and deposited in the body of juvenile and mature pigs, but the level of depositoin varied between different organs. Since *trans* fatty acids were absent or only present at very low levels in brain PE of pigs of all ages, the transfer of *trans* fatty acids through the blood-brain barrier must have been very limited. Moreover, no effects of *trans* fatty acids on nerve histology and function were found. PE of heart and liver mitochondria, and of lipids in adipose tissues and in plasma contained *trans* fatty acids at levels that reflected the dietary content, the level being somewhat lower in the former than in the two latter tissues.

The levels of *trans* fatty acids were generally lower in the tissues compared to that in the corresponding diets. The reason for this finding may be that the *trans* fatty acids were catabolized rather than deposited, but may also be due to the *trans* fatty acids, being diluted in the tissues with *de novo* synthesized *cis* monoenoic fatty acids. Since *trans* fatty acids were found in the tissues of the newborn pigs, transfer of *trans* fatty acids through the placenta must have occurred. *trans* Fatty acids were secreted into the milk with little discrimination and absorbed and deposited in the body of the suckling pig. Newborn pigs

from mothers fed *trans* fatty acids had markedly lower levels of *trans* fatty acids in their bodies than their mothers. The levels of *trans* fatty acids rose quickly during the first weeks of suckling and had reached the levels of the mature animals three weeks after birth. The depositions of 18:1 *trans* were similar in PHSBO and PHFO. The deposition of 20:1 *trans*, only present in PHFO, was markedly lower than that of 18:1 *trans* in PE, while the two isomers were deposited to a similar degree in adipose tissues.

The dietary content of linoleic acid had no consistent effect on the deposition of *trans* fatty acids. Dietary *trans* fatty acids had minor but consistent effects on the profile of essential fatty acids (*i.e.*, n-6 fatty acids) in the organs, causing a minor increase in the content of the lower homologs and a minor decrease in the content of the chain-elongated and desaturated higher homologs. All together, consumption of *trans* fatty acids from PHSBO and PHFO at levels of three times that consumed by humans in Western society showed no effects in the pigs that could be considered detrimental.

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Influence of Dietary Fatty Acids on the Glycerophospholipid Composition in Organs of Cod (*Gadus morhua*)

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Cod (mean start weight of 26 g) were fed three diets for 15 months, each based on a dry pellet coated at a level of 9 g/100 g with soybean oil, capelin oil or sardine oil. The fatty acid compositions of neutral lipids and four glycerophospholipids of white muscle, liver, gills and heart were determined. The fatty acid composition of dietary lipids influenced the composition of neutral lipids in all organs. Linoleic acid (18:2n-6) from soybean oil was selectively incorporated into phosphatidylcholine of the four tissues. Similar levels of 20:5n-3 and 22:6n-3 in phosphatidylcholine and phosphatidylethanolamine were found in all organs from cod fed capelin oil and sardine oil in spite of highly differentiated feed fatty acid levels. The polyunsaturated fatty acid (PUFA) composition of phosphatidylinositol was least influenced by dietary lipids. The preferred monoenoic fatty acid in phospholipids of cod was 18:1n-9, independent of dietary intake, whereas the longer chain monoenoic acids seemed to be preferentially catabolized. The results suggest that 20:4n-6 as well as 20:5n-3 and 22:6n-3 fatty acids are essential for cod.
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While it is known that the fatty acid composition of fish lipids generally reflects the dietary intake, the influence of dietary lipids differs between triacylglycerols and phospholipids, as has been shown for cod (1). However, detailed studies documenting the effect of the fatty acid composition of dietary lipids on the fatty acid composition of phospholipids in cod tissues have not been undertaken.

Polyunsaturated fatty acids (PUFA) in organs and tissues of marine fish are mainly of the n-3 series, whereas the n-6 PUFA are prominent in terrestrial animals. In the major phospholipids of marine animals, 20:5n-3 and 22:6n-3 are most prominent; however, phosphatidylinositol also contains high levels of 20:4n-6 (2–6).

The factors that govern the specific incorporation of the n-3 and n-6 fatty acids into membrane phospholipids, and the role of PUFA in the membranes, are not fully understood. The extent to which n-3 fatty acids can substitute for n-6 fatty acids in the biomembranes of mammals is of considerable interest. Many studies on blood platelets and tissue lipids confirm that long-chain n-3 fatty acids from diets are incorporated into membrane lipids (7). Variations in the composition of polyunsaturated fatty acids in the diet effectively modulate eicosanoid biosynthesis, which generally depends on the composition of the fatty acid precursor pool (8,9).

In the present study, we examined the influence of dietary fatty acids on the composition of individual glycerophospholipid classes in tissues of cod and the ability of cod to modify the fatty acids taken up from the diet.

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Abbreviations: EFA, essential fatty acids; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; TAG, triacylglycerols; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Fish and diets. Cod, average weight 26 ± 2 g, hatched and reared at the Institute of Marine Research, Austevoll Aquaculture Research Station (N-5392 Storebø, Norway) were placed in 3 sea cages (380 fish in each) in November 1988. The fish were fed *ad libitum* once daily pellets of dry feed (produced by T. Skretting, Stavanger, Norway) which contained per 100 g: 36 g fishmeal ("Norseamink" and "LT-meal"; Norsildmel, Bergen, Norway), 32 g concentrated soybean meal (Danpro A, Aarhus Oliefabrik A/S, Århus, Denmark), 16 g wheat flour and 6 g mineral and vitamin premixes. The dry pellets were coated with 9 g/100 g of soybean oil (diet A), capelin oil (diet B) or sardine oil (diet C). The oils were supplemented with vitamin E (α-tocopheryl acetate) and L-ascorbic acid (suspended in the oil to avoid loss in processing) to give final feed concentrations of 300 mg/kg vitamin E and 500 mg/kg ascorbic acid, respectively. The mean analyzed lipid content of the three feeds was 10.5%. Feed batches of 15 kg were prepared several times during the feeding period. The fish were studied from a start weight of 26 g to maturity, a period of 15 mon, and had then reached an average weight of 980 g ± 200 g. Five fish from each group were collected in March 1990, anaesthetized with benzocaine and killed by a sharp blow on the head. Pooled samples of white muscle, liver, gill and heart were collected. The samples were immediately frozen on dry ice (−80°C), and kept at this temperature until analyses were carried out.

Analytical methods. The organs were homogenized and extracted. The neutral lipids and the glycerophospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) were separated according to Lie and Lambertsen (10). High-performance liquid chromatography (HPLC) was done using a Constametric II pump, a steel column (25 × 0.46 cm) equipped with a Rheodyne (7125) sample injection valve with a 20-μL loop, and a variable wavelength spectrophotometer (LDC Spectromonitor III). Stepwise elution was achieved by means of a Rheodyne six-position rotary valve. The column was packed with silica gel (LiChrosorb 5 μm, Merck, Darmstadt, Germany) using a Maximator pump (MSE 72, Schmidt, Kranz & Co. GmbH, Zorge/Südharz, Germany). All solvents were of HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland). The flow rate was maintained at 1 mL/min and elution was monitored at 205 nm.

The identity and purity of the phospholipids isolated were verified by thin-layer chromatography (TLC; Merck, Kieselgel 60) by comparison with standard phospholipids. PC, PE, PI and PS were from Sigma (St. Louis, MO); lysophosphatidylcholine (LysoPC) was from Serdary Research Laboratories (London, Canada). The solvent system was ethyl acetate/*n*-propanol/chloroform/methanol/0.25% aqueous KCl (15:25:25:10:4.5, by vol).

The fractions were evaporated in a block heater with a stream of N_2; 19:0 fatty acid was added as an internal standard. The fatty acids were trans esterified in 12% BF_3 in methanol. The methyl esters were separated on a Carlo Erba 2900 gas chromatograph using on column injection with a 50-m, 0.3 mm i.d., CP-sil 88 fused silica capillary column (Chrompack, Middelburg, The Netherlands). The fatty acid composition was calculated using a Maxima 820 (Chromatography Workstation, installed in an IBM-AT), connected to the gas chromatograph. Peaks were identified by reference to standard mixtures of methyl esters (Nu-Chek, Elysian, MN).

RESULTS

Weight gains, feed intakes, liver weights and hepatosomatic indices were not significantly different among the dietary treatments. The fatty acid compositions of the experimental diets are given in Table 1, which show that the n-3 fatty acids accounted for 15, 20 and 30% of the sum, in feeds A, B and C, respectively. In the liver, the fatty acid composition of the neutral lipids was similar to that of the feed lipids, except for lower levels of 20:1n-9 and 22:1n-11, and higher levels of 18:1n-9 (Table 2). The n-3 fatty acid levels in the neutral lipids in the liver and the feed were similar, but the level of 22:6n-3 was higher in the liver in groups B and C than in the feed. Linoleic acid (18:2n-6) accumulated in all phospholipids in the liver from group A, while the levels of n-3 fatty acids were lower in this group than in the PC fractions from the two other groups. The high levels of 22:1n-11 and 20:1n-9 in feed B (30%) were incorporated into the liver glycerophospholipids only to the extent of 8% and the major monoenoic acid in group B was 18:1n-9, as it was in groups A and C. In all groups, 22:6n-3 was the major n-3 fatty acid in the phospholipids.

Table 3 details the fatty acid composition of the neutral lipids and of the four glycerophospholipids in the muscle tissue from the three groups. The linoleic acid from diet A was heavily incorporated into all muscle lipids. The levels of n-3 fatty acids and n-3/n-6 ratios in neutral lipids corresponded to those of the lipids in the diets. The glycerophospholipids of group A had the lowest n-3 levels, and the n-3 level of the glycerophospholipids of group B and C were at the same high levels, *i.e.*, 40–60%. The content of 18:3n-3 in the dietary lipid of group A was reflected in the composition of the neutral lipids, of PC and of PE. The level of 18:4n-3 in the diet B lipids was reflected in the neutral lipids of this group. All lipids of group C showed elevated levels of 20:4n-6.

The fatty acid composition of neutral lipids and individual phospholipids in the heart is shown in Table 4. The fatty acid composition of the neutral lipids was to some extent altered by the dietary lipids, as exemplified by the n-3/n-6 ratio. The levels of 20:5n-3 and 22:6n-3 were high in all lipids from the three groups and seemed little affected by the dietary lipid composition. The levels of arachidonic acid in heart PI were 12.3% in group A, 15.3% in group B, and 20.5% in group C.

Table 5 details the fatty acid composition of neutral lipids and individual phospholipid classes from the gills. As for the other tissues, linoleic acid was heavily incorporated into all lipid classes of group A, particularly into neutral lipids and PC. The values for 20:4n-6, 20:5n-3 and 22:6n-3 were quite similar in gill phospholipids from all three groups regardless of the dietary lipid composition. High levels of 20:2n-6 (1.4–2.6%) were found in the neutral lipids as well as in the individual phospholipids of the gills of cod fed diet A.

TABLE 1

Fatty Acid Composition of the Experimental Diets

Fatty acid[a]	Dietary oil		
	Soybean	Capelin	Sardine
14:0	3.3	6.1	6.7
16:0	12.6	12.1	14.8
18:0	2.7	1.3	2.1
Σ Saturated	18.7	20.0	24.1
16:1n-9	—	0.2	0.3
16:1n-7	4.3	7.4	7.6
18:1n-11	—	—	—
18:1n-9	16.2	9.3	9.6
18:1n-7	2.2	2.3	2.9
20:1n-11	0.3	—	0.8
20:1n-9	5.5	13.2	6.3
22:1n-11	7.1	16.5	8.0
Σ Monounsaturated	36.7	52.0	37.5
18:2n-6	28.8	5.2	4.1
18:3n-3	3.9	1.2	1.0
18:4n-3	1.4	3.5	2.7
20:2n-6	—	0.2	—
20:4n-6	0.1	0.2	0.8
20:5n-3	4.5	7.7	13.0
22:5n-3	0.4	0.6	1.6
22:6n-3	4.5	6.8	10.8
Σ n-6	29.0	5.6	5.0
Σ n-3	15.0	20.3	29.8
n-3/n-6 Ratio	0.5	3.6	6.0

[a]Expressed as percentages (w/w) of total fatty acids present: —, less than 0.1%.

DISCUSSION

Several reviews discuss the requirement of essential fatty acids (EFA) for fish (11–14). In the present study the fish behaved normally and no apparent signs of nutritional imbalance or pathology were observed during the feeding period, indicating that the dietary level of EFA was adequate to allow normal growth in all groups.

Cod stores triacylglycerols (TAG) exclusively in the liver, which may reach a fat content of 60–70%; the relative weight of this organ may increase from 5 to 15% as a result of excessive feeding or of a high fat diet (1,15). The fatty acid composition of the dietary lipids has been shown to have a pronounced effect on the composition of liver triacylglycerols, and also to affect the composition of polar lipid fatty acids in liver and muscle (1). The fatty acid composition of the neutral lipids in the liver, consisting mainly of triacylglycerols, confirms that cod stores the feed lipids with only minor modifications, such as preferring 22:6n-3 over 20:5n-3, and some discrimination against 20:1 and 22:1 isomers.

The fatty acid composition of the individual phospholipids in liver, fillet, heart and gills from cod was generally

TABLE 2

The Fatty Acid Composition of Neutral Lipid (N), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI) and Phosphatidylserine (PS) in Liver of Cod Fed Diets Containing Soybean Oil, Capelin Oil or Sardine Oil

	Soybean oil					Capelin oil					Sardine oil				
Fatty acid[a]	N	PC	PE	PI	PS	N	PC	PE	PI	PS	N	PC	PE	PI	PS
14:0	1.9	1.5	1.2	2.0	3.7	3.8	1.8	1.4	1.9	2.3	3.3	2.1	1.2	1.3	1.4
16:0	10.9	23.1	9.5	13.6	19.1	13.2	25.7	9.5	11.8	14.9	12.7	27.6	10.3	11.1	14.9
18:0	3.5	3.6	6.8	13.2	10.0	6.3	2.5	5.8	13.8	12.2	3.6	2.2	6.5	12.5	8.9
Σ Saturated	16.7	29.1	18.4	31.0	35.8	20.5	30.9	17.9	29.9	32.4	20.1	32.7	18.8	26.4	27.4
16:1n-9	0.4	1.2	0.8	2.7	3.9	0.5	1.1	0.7	1.1	1.3	0.6	1.1	0.7	1.2	1.0
16:1n-7	2.7	0.9	1.4	1.4	1.3	6.3	1.1	2.2	2.1	2.0	5.7	1.2	2.2	2.6	2.9
18:1n-11	—	0.7	1.3	—	—	2.0	1.1	2.7	1.3	0.5	1.2	0.5	1.1	1.4	1.2
18:1n-9	24.0	12.6	16.0	18.5	17.2	17.8	9.3	14.0	15.1	11.3	17.9	8.6	15.1	15.4	15.9
18:1n-7	2.8	2.0	3.5	2.4	2.3	3.9	2.2	7.8	3.0	3.3	4.7	2.1	7.9	3.7	3.7
20:1n-11	0.7	—	0.2	0.2	—	1.7	—	0.7	0.6	0.3	1.2	—	0.6	0.8	0.8
20:1n-9	3.1	0.9	2.3	1.7	1.9	9.7	1.0	5.7	4.0	4.6	3.6	0.4	3.1	3.5	4.7
22:1n-11	2.2	0.2	1.0	1.0	—	7.2	—	2.3	2.2	0.9	2.7	—	1.4	2.1	2.7
Σ Monounsaturated	36.1	18.4	26.6	27.8	26.6	50.9	16.0	36.6	29.5	24.2	38.0	13.9	32.4	30.8	32.9
18:2n-6	33.6	15.7	23.9	20.0	17.3	6.7	3.3	6.6	13.3	7.5	6.1	2.1	6.6	8.6	11.8
18:3n-3	3.9	1.1	2.2	2.1	1.6	1.3	0.4	0.8	1.4	0.5	1.2	0.3	0.8	1.2	1.5
18:4n-3	0.8	—	0.2	0.2	0.2	2.3	0.3	0.8	0.8	0.5	1.8	0.1	0.8	1.0	1.0
20:2n-6	0.5	0.2	0.7	0.2	0.3	0.3	—	0.4	—	—	0.3	—	0.3	—	0.4
20:4n-6	—	0.6	1.0	4.1	0.7	0.2	0.6	0.9	5.7	1.0	0.7	1.4	2.0	11.0	0.6
20:5n-3	3.0	10.0	8.9	6.5	3.5	6.6	14.9	13.6	8.0	4.7	12.5	15.1	14.5	8.2	7.0
22:5n-3	0.4	1.0	0.9	0.8	0.6	0.8	1.2	1.1	1.0	0.9	2.1	1.3	1.5	1.5	1.2
22:6n-3	3.9	23.4	16.7	7.2	11.4	7.6	30.3	19.5	9.2	18.8	12.5	32.4	20.0	10.0	15.4
Σ n-6	34.2	16.5	25.6	24.3	18.3	7.2	3.8	7.9	19.0	8.5	7.1	3.5	9.0	19.6	12.7
Σ n-3	12.2	35.7	29.0	16.9	17.3	19.2	47.6	36.4	20.6	25.4	30.8	49.5	38.1	22.3	26.5
n-3/n-6 Ratio	0.4	2.2	1.1	0.7	1.0	2.7	12.5	4.6	1.2	3.5	4.3	14.1	4.3	1.1	2.1

[a]Expressed as percentages (w/w) of total fatty acids present; —, less than 0.1%.

TABLE 3

The Fatty Acid Composition of Neutral Lipid (N), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI) and Phosphatidylserine (PS) in Fillet of Cod Fed Diets Containing Soybean Oil, Capelin Oil or Sardine Oil

	Soybean oil					Capelin oil					Sardine oil				
Fatty acid[a]	N	PC	PE	PI	PS	N	PC	PE	PI	PS	N	PC	PE	PI	PS
14:0	2.5	0.5	0.6	0.5	0.9	2.4	0.9	0.3	0.2	1.2	2.2	0.9	0.3	0.9	0.9
16:0	14.4	23.5	7.6	4.4	8.6	15.1	26.7	7.6	4.8	11.5	16.6	25.4	7.5	5.9	9.6
18:0	3.9	1.0	5.3	32.4	22.7	3.1	0.9	4.7	30.7	15.0	4.7	1.1	7.4	31.1	16.7
Σ Saturated	21.2	25.5	13.9	38.2	36.5	20.9	29.1	13.1	37.0	30.5	23.6	27.8	15.3	39.0	30.1
16:1n-9	0.6	0.3	0.6	0.4	1.3	0.5	0.3	0.2	0.4	1.2	0.6	0.4	0.2	0.6	0.9
16:1n-7	3.7	0.5	0.3	0.3	—	4.0	1.1	0.4	0.6	1.2	3.5	0.8	0.4	0.8	0.6
18:1n-11	1.1	0.3	0.2	0.2	0.4	1.4	0.9	1.5	0.9	1.5	0.9	0.5	0.7	0.2	0.8
18:1n-9	16.6	7.2	6.7	5.4	7.4	15.0	6.3	5.8	5.7	9.6	12.1	6.6	6.2	5.7	8.9
18:1n-7	4.0	1.1	2.0	1.0	1.7	4.5	1.6	3.7	1.8	3.0	5.4	2.0	4.0	1.8	3.7
20:1n-11	0.8	—		0.2	0.1	—	0.5	0.1	0.4	0.1	0.3	0.7	0.1	0.2	—
20:1n-9	3.8	0.3	1.2	0.4	2.3	5.5	0.9	3.2	0.9	5.6	3.1	0.5	1.7	0.9	3.2
22:1n-11	2.6	—	0.1	0.2	—	4.3	—	0.3	0.4	1.0	1.8	—	0.1	0.5	0.2
Σ Monounsaturated	33.2	9.9	11.6	8.0	13.2	36.5	11.7	16.0	10.8	23.7	28.1	11.3	13.8	10.7	18.6
18:2n-6	17.5	25.7	14.9	5.0	6.3	11.7	5.6	4.4	2.8	5.6	7.6	3.3	3.7	1.8	2.3
18:3n-3	2.1	1.8	1.1	0.4	0.4	1.4	0.6	0.4	0.2	0.6	1.0	0.4	0.3	0.2	—
18:4n-3	1.3	0.3	0.1	0.1	—	1.7	0.8	0.2	0.1	0.3	1.3	0.4	0.1	0.2	—
20:2n-6	0.6	0.3	0.5	0.1	0.7	0.5	0.1	0.3	—	0.1	0.4	0.1	0.2	—	0.4
20:4n-6	0.5	0.9	1.3	3.7	0.2	0.6	1.2	1.1	3.8	—	1.6	3.3	3.1	5.1	1.1
20:5n-3	8.6	12.1	13.7	8.4	2.8	10.1	22.0	15.9	10.6	3.9	13.4	22.9	15.2	7.5	5.1
22:5n-3	1.2	1.1	1.8	1.1	1.5	1.2	1.3	2.1	1.4	1.8	1.6	1.7	2.9	1.6	2.8
22:6n-3	12.3	21.4	38.5	34.1	34.3	14.6	26.3	43.8	33.1	32.9	19.8	26.9	41.2	33.3	37.0
Σ n-6	18.6	26.9	16.7	8.8	7.2	12.7	6.9	5.8	6.6	5.7	9.6	6.7	7.0	6.9	3.8
Σ n-3	26.0	37.0	55.6	44.2	39.1	29.4	51.5	63.2	45.4	39.5	37.6	52.6	60.3	43.0	45.1
Σ-3/n-6 Ratio	1.4	1.4	3.3	5.0	5.4	2.5	7.5	10.9	7.0	8.2	3.9	7.8	8.6	6.2	11.7

[a]Expressed as percentages (w/w) of total fatty acids present; —, less than 0.1%.

TABLE 4

The Fatty Acid Composition of Neutral Lipid (N), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI) and Phosphatidylserine (PS) in Heart of Cod Fed Diets Containing Soybean Oil, Capelin Oil or Sardine Oil

	Soybean oil					Capelin oil					Sardine oil				
Fatty acid[a]	N	PC	PE	PI	PS	N	PC	PE	PI	PS	N	PC	PE	PI	PS
14:0	1.3	0.7	0.3	0.8	1.0	2.4	1.4	0.2	0.7	0.6	2.4	1.3	0.1	0.7	0.7
16:0	11.4	23.5	3.4	6.8	7.1	11.9	25.0	3.4	5.1	6.0	13.5	25.2	3.4	5.9	6.0
18:0	5.0	3.4	9.8	27.7	21.1	4.6	2.9	10.2	29.8	18.6	5.0	3.0	12.1	30.3	19.3
Σ Saturated	17.7	27.8	14.1	36.5	30.1	19.0	29.8	14.0	36.7	27.4	21.0	30.2	16.1	38.8	28.2
16:1n-9	—	0.4	0.1	1.8	1.5	0.3	0.4	0.1	0.6	0.9	—	0.5	0.2	1.5	1.3
16:1n-7	1.6	0.5	0.2	—	0.4	4.0	0.9	0.2	0.5	0.1	4.0	0.9	0.1	—	—
18:1n-11	0.4	0.4	0.3	—	0.2	1.7	0.9	0.9	0.9	0.9	1.0	0.2	0.4	—	0.3
18:1n-9	13.9	16.5	5.6	7.8	4.1	14.3	14.6	5.1	6.3	3.2	14.7	14.4	4.9	6.8	3.5
18:1n-7	4.2	2.0	3.5	1.8	2.9	5.1	3.0	7.0	2.9	4.2	6.3	3.2	7.5	3.0	5.0
20:1n-11	—	0.1	0.1	—	0.3	0.4	0.6	0.2	—	0.6	—	0.4	0.1	—	—
20:1n-9	2.0	0.8	1.8	0.2	4.5	5.6	1.7	3.9	1.1	8.9	2.3	0.8	1.7	0.2	5.1
22:1n-11	1.6	—	0.3	—	0.8	4.7	0.1	0.2	—	0.3	1.7	—	—	—	0.1
Σ Monounsaturated	23.7	20.9	12.2	11.7	14.7	36.5	22.9	18.3	12.5	19.4	30.2	20.9	15.1	11.6	15.3
18:2n-6	27.6	15.4	11.0	7.8	3.2	11.3	4.0	3.2	2.2	1.4	7.6	2.6	2.6	1.6	1.2
18:3n-3	2.7	1.1	1.1	0.2	0.3	1.5	0.4	0.4	—	—	1.0	0.3	0.3	—	—
18:4n-3	0.3	0.1	—	—	—	1.4	0.4	0.1	—	—	1.2	0.2	—	—	—
20:2n-6	0.4	0.8	1.1	0.2	1.7	—	0.4	0.5	—	0.8	—	0.2	0.5	—	0.8
20:4n-6	1.6	1.3	2.4	12.3	0.9	1.3	1.4	2.6	15.3	0.8	2.6	3.0	5.0	20.5	1.9
20:5n-3	7.4	10.1	10.6	18.4	4.8	8.9	13.9	12.5	19.4	4.3	12.0	13.4	10.7	14.6	4.1
22:5n-3	1.0	1.0	2.1	0.4	2.7	1.2	1.1	1.9	0.7	2.2	1.8	1.4	2.3	0.9	2.5
22:6n-3	17.5	20.9	39.9	10.7	37.7	19.0	24.0	42.0	11.3	41.1	22.5	25.9	43.6	11.3	42.4
Σ n-6	29.6	17.5	14.5	20.2	5.7	12.6	5.8	6.5	17.5	3.0	10.2	5.9	8.2	22.1	3.9
Σ n-3	29.0	33.3	54.2	29.8	45.5	31.9	40.3	57.9	31.5	47.6	38.6	41.5	57.5	26.7	48.9
n-3/n-6 Ratio	1.0	1.9	3.7	1.5	8.0	2.5	7.0	8.9	1.8	16.1	3.8	7.1	7.0	1.2	12.4

[a]Expressed as percentages (w/w) of total fatty acids present; —, less than 0.1%.

TABLE 5

The Fatty Acid Composition of Neutral Lipid (N), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI) and Phosphatidylserine (PS) in Gills of Cod Fed Diets Containing Soybean Oil, Capelin Oil or Sardine Oil

	Soybean oil					Capelin oil					Sardine oil				
Fatty acid[a]	N	PC	PE	PI	PS	N	PC	PE	PI	PS	N	PC	PE	PI	PS
14:0	1.6	1.2	0.3	1.7	0.4	2.6	1.8	0.4	2.1	0.8	3.3	1.5	0.3	2.3	0.6
16:0	12.7	24.2	6.7	9.9	6.5	14.7	27.3	6.9	13.2	8.8	14.2	26.2	6.9	12.9	7.9
18:0	5.8	3.5	7.2	25.0	20.2	6.2	2.7	7.5	23.2	17.9	5.3	2.8	9.0	23.6	17.9
Σ Saturated	20.8	29.6	15.1	38.6	30.4	24.9	33.0	17.7	41.2	32.0	24.3	32.1	19.6	40.9	32.0
16:1n-9	0.9	1.6	0.4	2.7	0.6	0.9	1.3	0.3	2.9	1.3	0.8	1.6	0.4	2.4	1.1
16:1n-7	2.2	1.5	0.2	0.4	0.3	3.9	2.6	0.5	0.7	0.6	4.6	2.5	0.4	0.9	0.5
18:1n-11	—	—	—	—	—	1.4	0.7	1.0	0.5	0.4	0.5	0.2	0.5	1.0	—
18:1n-9	17.8	18.0	9.3	8.4	6.6	14.2	18.0	6.9	10.6	6.8	12.5	17.5	6.9	9.2	6.3
18:1n-7	3.0	2.0	2.9	1.8	2.8	4.5	3.1	5.9	4.0	5.0	4.7	3.4	8.9	3.9	6.7
20:1n-11	0.3	0.3	—	—	—	0.9	0.6	0.2	—	0.2	0.6	0.4	—	0.2	—
20:1n-9	3.4	1.6	2.6	1.2	4.5	6.8	2.5	4.8	2.4	6.9	4.2	1.5	2.9	2.0	4.5
22:1n-11	1.7	—	—	0.2	0.1	4.4	0.2	0.2	0.2	0.3	3.2	—	—	0.5	—
Σ Monounsaturated	29.8	25.1	15.4	14.7	15.1	38.4	29.7	20.1	21.3	19.8	32.2	27.6	20.8	20.5	19.8
18:2n-6	22.2	14.9	8.8	6.4	5.3	5.0	3.1	2.1	3.4	1.6	3.7	2.1	1.8	2.9	1.1
18:3n-3	2.1	0.8	0.4	0.2	0.2	0.6	0.3	—	—	—	0.6	0.2	—	0.2	—
18:4n-3	0.6	0.2	—	—	—	1.1	0.4	—	—	—	1.3	0.2	—	0.3	—
20:2n-6	1.6	1.8	2.3	1.4	2.6	0.7	0.6	0.8	0.3	0.8	0.5	0.5	0.7	0.2	0.9
20:4n-6	1.4	0.8	1.7	11.9	0.9	2.2	1.3	2.4	9.1	1.0	3.3	3.0	4.9	12.6	2.3
20:5n-3	6.4	8.0	8.9	9.9	4.0	9.3	11.5	11.1	10.3	5.0	12.2	12.1	10.0	9.1	4.9
22:5n-3	0.8	0.9	1.7	0.7	1.2	1.0	1.2	2.1	0.8	1.2	1.7	1.5	2.5	1.2	1.5
22:6n-3	12.9	16.4	36.0	11.6	37.7	14.5	16.9	34.6	10.6	31.6	17.5	18.6	32.7	10.5	34.4
Σ n-6	25.3	17.6	12.7	19.6	8.9	7.8	4.9	5.3	12.8	3.5	7.4	5.5	7.4	15.7	4.3
Σ n-3	22.8	26.6	47.2	22.3	43.1	27.1	30.7	48.2	21.7	37.9	33.8	32.9	45.4	21.6	40.8
n-3/n-6 Ratio	0.9	1.5	3.7	1.1	4.8	3.5	6.2	9.1	1.7	10.8	4.5	6.0	6.2	1.4	45.1

[a]Expressed as percentages (w/w) of total fatty acids present; —, less than 0.1%.

in good agreement with previous findings (2,4,10,16). The fatty acid composition of the phospholipids is important for the function of biomembranes. The balance between saturated, monounsaturated and polyunsaturated fatty acids maintains the so-called fluidity of the biomembranes, and the requirement of n-3 PUFA in fish is directly related to their role as components of membrane phospholipids (17). The incorporation of 18:2n-6 into PC of fillet, heart and gill in cod fed diet A was only partly balanced by a corresponding reduction in 20:5n-3 and 22:6n-3. Hence, 18:2n-6 may be incorporated into PC as an alternative to monoenoic or saturated fatty acids, and may be less "essential" for cod phospholipids. In spite of a 50% higher intake of 20:5n-3 and 22:6n-3 in feed C compared to feed B, cod fed these diets had similar levels of the two fatty acids in PC and PE, suggesting that an optimum level of n-3 was achieved in cod fed diet B.

Interestingly, PI (but to some extent also PS) PUFA composition in cod tissues were least influenced by dietary intake, which warrants special attention. The fatty acid composition of fish PI, having a high content of the molecular species 18:0/20:4n-6, resembles that of terrestrial animals, where PI has been shown to play a critical role in the transduction of hormone signals and in the provision of precursors for eicosanoid synthesis (18,19). PI in cod fed the highest level of 20:5n-3 (and of 20:4n-6) had the lowest 20:5n-3/20:4n-6 ratio in all tissues, and particular attention should therefore be given to the ratio 20:5n-3/20:4n-6 when studying fish eicosanoid synthesis. The ratio of 20:5n-3/20:4n-6 in phospholipids of mammals has been used as an index of the availability of precursors for platelets proaggregatory compounds (20,21). Henderson *et al.* (22) reported a higher rate of prostaglandin synthesis in gills than in liver. The capacity of gill tissue of aquatic species to form prostaglandins indicates a potential role for these compounds in controlling osmoregulation and perhaps respiratory processes. The regulated PI composition in the cod gills in this experiment points to the importance of the fatty acid composition for the function of the gills.

All feed lipids contained substantial amounts of 20:1 and 22:1, due to the fish meal lipids. In particular, diet B had very high levels of the two fatty acids (13.2% and 16.5%, respectively) from capelin oil in the feed. Lie and Lambertsen (23) reported considerably lower levels compared to the contents in the diets of these acids and higher levels of 18:1n-9 in the intestinal wall as well as in chylomicra, and pointed to the role of intestinal cells in the catabolism of 22:1n-11 and possibly of 20:1n-9. The presence of only minor amounts of n-11 monoene isomers of shorter chain length (17,19,21) indicates that a peroxisomal chain shortening, as shown for rat tissues, is not a major pathway for disposing of ingested 22:1n-11. Cod tissues may have specific enzymes for the catabolism of 22:1n-11 to respiratory products or acetyl CoA, the latter being used for *de novo* synthesis of 16:0 and 18:1n-9. Neither 22:1n-11 nor 20:1n-9 are prominent in the glycerophospholipids of cod, with the exception of the levels found in PS in the erythrocytes (5). The preferred monoenoic fatty acid in phospholipids of cod evidently is 18:1n-9, independent of the dietary intake.

Fish are capable of modifying dietary as well as endogenous fatty acids by desaturation and elongation. The ability of fish to modify dietary n-6 and n-3 fatty acids has been demonstrated for some species, particularly for salmonids (24,25; Lie, Waagbø and Sandnes, unpublished observation). In fish as in mammals, the substrate preference for Δ6 fatty acids desaturase decreases in the order 18:3n-3 > 18:2n-6 ⩾ 18:1n-9 (26,27). Cod fed diet A contained more 18:3n-3 than did the other groups in all tissues and in the individual phospholipids examined, suggesting that little or no 18:3n-3 was desaturated and elongated to C_{20} and C_{22} PUFA. This was also true for 18:4n-3 in cod fed diet B. As already discussed, 18:2n-6 was incorporated into all phospholipids analyzed, and high levels were observed in all tissues of cod fed diet A and only trace levels of 18:3n-6, 20:2n-6 and 20:3n-6 were found. Diet C, however, contained the highest concentration of 20:4n-6 (although only 0.8%), and with the exception of gill PI, the tissue phospholipids of the cod fed this diet had the highest levels of arachidonic acid.

The present feeding experiments thus indicate that very limited enzyme activity exists in cod to modify C_{18} PUFA, n-6 as well as n-3, except perhaps for the gills, when adequate amounts of n-3 PUFA are fed. Leger *et al.* (28), concluded that the longer chain n-3 PUFA as 22:6 inhibit Δ6 desaturase in rainbow trout, preventing desaturation of both 18:2n-6 and 18:3n-3. Cod seems to convert 20:5 to 22:6 in all tissues, as generally higher levels of 22:6 were found. This was also seen in group C which was fed a diet having a higher level of 20:5 than of 22:6.

The present results suggest that 20:4n-6, 20:5n-3 and 22:6n-3 are essential for normal growth in cod and also further the preferential catabolism of the longer chain monoenoic acids (20:1n-9 and 22:1n-11).

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Two Unsaturated 9*R*-Hydroxy Fatty Acids from the Cyanobacterium *Anabaena flos-aquae f. flos-aquae*

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(9*R*-10*E*,12*Z*,15*Z*)-9-Hydroxyoctadecatrienoic acid and (9*R*,10*E*,12*Z*)-9-hydroxyoctadecadienoic acid were isolated from the nitrogen fixing cyanobacterium *Anabaena flos-aquae f. flos-aquae* and characterized as the corresponding methyl esters. This is the first report of the natural occurrence of 9*R*-oxygenated fatty acids.
Lipids 27, 776–778 (1992).

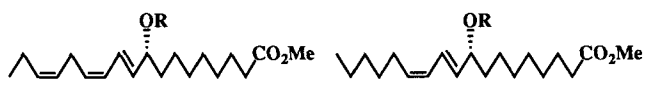
Our previous work had shown that cyanobacteria contain glyceroglycolipids in high amounts and that their fatty acid distribution is different from that of higher plants (1,2). Furthermore, we characterized the novel galactolipid, 1,6′-*O*-diacyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol, from the cyanobacterium *Anabaena flos-aquae f. flos-aquae* (3). As a continuation of our investigation of lipophilic constituents in *A. flos-aquae f. flos-aquae,* we have now isolated two 9*R*-hydroxy unsaturated fatty acids and characterized them as their methyl esters.

EXPERIMENTAL PROCEDURES

General. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a JEOL (Akishima, Japan) EX-270 (270 MHz) or GSX-400 (400 MHz) spectrometer using tetramethylsilane (TMS) as internal standard. Infrared (IR) spectra were obtained with a Perkin-Elmer (Norwalk, CT) 1650QS Fourier transform IR (FTIR) spectrometer. Ultraviolet (UV) spectra were recorded with a Shimadzu (Kyoto, Japan) UV-2100 spectrometer. Optical rotations were measured on a JASCO (Tokyo, Japan) DIP-140 digital polarimeter. Circular dichroism (CD) spectra were obtained with a JASCO J-600 spectrometer. High-performance liquid chromatography (HPLC) was carried out using a JASCO 880-PU pump equipped with a Shodex RI (Tokyo, Japan), SE-11 differential refractometer. For gas chromatography/mass spectrometry (GC/MS), a JEOL D-300 mass spectrometer interfaced to a Hewlett-Packard (Palo Alto, CA) 5710A gas chromatograph with a JEOL JMA 2000 data processing system was used. Thin-layer chromatography was done on Merck (Darmstadt, Germany) precoated Kieselgel 60F_{254} plates and fractions were detected by illumination with an ultraviolet lamp, or by spraying the plate with either 5% vanillin/conc.H_2SO_4 or 1% $Ce(SO_4)_2$/10% H_2SO_4 followed by heating. Column chromatography was carried out on silica gel BW-200 or BW-300 (Fuji Davison Chemicals Co., Ltd., Kasugai, Japan).

Culture conditions. The strain of *Anabaena flos-aquae f. flos-aquae* (NIES-74) used for the present study was provided by the National Institute for Environment Studies. *A. flos-aquae f. flos-aquae* was cultured in eight 5-L Erlenmeyer flasks containing CB medium. Cultures were illuminated continuously at 1500 lux with cool-white fluorescent lamps and vigorously aerated with sterilized air passed through a 0.2 μm membrane filter at a rate of 0.5 L/min. After three weeks, the alga was harvested by centrifugation at 20,000 × *g* from the combined 40-L culture and lyophilized.

Extraction and isolation. Lyophilized alga (8.02 g) was homogenized in $CHCl_3$/MeOH (1:1, vol/vol) and extracted at room temperature three times for 12 h. The resulting extract (1.16 g) was successively subjected to silica gel column chromatography ($CHCl_3$/MeOH, 20:1→10:1, vol/vol) to afford a fraction containing the free acids of 1 and 2.



1: R=H
1a: R=Bz
1b: R=(+)-MTPA

2: R=H
2a: R=Bz
2b: R=(+)-MTPA

The fraction was treated with etheral diazomethane to prepare the corresponding methyl esters, which were purified by silica gel column chromatography (*n*-hexane/EtOAc, 5:1, vol/vol) followed by HPLC (Nucleosil 50-5; Gasukuro Kogyo Inc., Tokyo, Japan; *n*-hexane/EtOAc, 88:12, vol/vol) to furnish 1 (7.9 mg) and 2 (1.5 mg).

1: A colorless oil. $[\alpha]_D^{23}$ −10.8° (c = 0.39, EtOH). UV λ_{max}^{MeOH}nm (ε): 235 (28100). IR ν_{max}^{film} cm^{-1}: 3427, 1738, 988. ^{1}H NMR ($CDCl_3$, 270 MHz, δ): 0.98 (3H, *t*, *J* = 6.6 Hz, 18-H_3), 2.08 (2H, *m*, 17-H_2), 2.30 (2H, *t*, *J* = 7.4 Hz, 2-H_2), 2.93 (2H, *dd*, *J* = 7.4, 7.4 Hz, 14-H_2), 3.67 (3H, *s*, CO_2CH_3), 4.16 (1H, *dt*, *J* = 6.6, 6.6 Hz, 9-H), 5.32 (1H, *m*, 15-H), 5.42 (2H, *m*, 13-H, 16-H), 5.69 (1H, *dd*, *J* = 6.6, 15.2 Hz, 10-H), 5.99 (1H, *dd*, *J* = 11.0, 11.0 Hz, 12-H), 6.51 (1H, *dd*, *J* = 11.0, 15.2 Hz, 11-H). Assignments for 13-H, 15-H, 16-H were confirmed by homonuclear decoupling as follows: 5.32 (1H, *dtt*, *J* = 1.5, 7.4, 10.6 Hz, 15-H), 5.41 (1H, *dddt*, *J* = 1.1, 1.5, 7.4, 11.0 Hz, 13-H), 5.42 (1H, *dt*, *J* = 7.0, 10.6 Hz, 16-H). MS *m/z* (%): 308 (M^+, 1.6), 290 ($M^+ - H_2O$, 44), 185 (12), 161 (15), 147 (38), 133 (51), 119 (42), 108 (80), 105 (56), 91 (77), 79 (100). High resolution MS: Found, 308.235; Calcd. for $C_{19}H_{32}O_3$ (M^+), 308.235.

2: A colorless oil. $[\alpha]_D^{23}$ −5.8° (c = 0.32, EtOH). UV λ_{max}^{MeOH}nm (ε): 233 (21000). IR ν_{max}^{film} cm^{-1}: 3420, 1739, 986. ^{1}H NMR ($CDCl_3$, 270 MHz, δ): 0.89 (3H, *t*, *J* = 6.6 Hz, 18-H_3), 2.18 (2H, *dt*, *J* = 7.8, 7.8 Hz, 14-H_2), 2.30 (2H, *t*, *J* = 7.6 Hz, 2-H_2), 3.66 (3H, *s*, CO_2CH_3), 4.15 (1H, *m*, 9-H), 5.45 (1H, *dt*, *J* = 7.8, 10.9 Hz, 13-H), 5.66 (1H, *dd*, *J* = 6.9, 15.2 Hz, 10-H), 5.97 (1H, *dd*, *J* = 10.9, 11.2 Hz, 12-H), 6.49 (1H, *dd*, *J* = 11.2, 15.2 Hz, 11-H). MS *m/z* (%): 310 (M^+, 4.6), 292 ($M^+ - H_2O$, 69), 185 (29), 163 (20), 153 (31), 135 (31), 121 (40), 107 (52), 93 (100), 79 (94). High

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Abbreviations: CD, circular dichroism; EI-MS, electron impact mass spectrometry; FTIR, Fourier transform infrared; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; HR-EIMS, high resolution electron impact mass spectrometry; IR, infrared; NMR, nuclear magnetic resonance; TMS, trimethylsilyl; UV, ultraviolet.

resolution MS: Found, 310.254; Calcd. for $C_{19}H_{34}O_3$ (M^+), 310.257.

Positional determination of the hydroxyl groups in 1 and 2. A solution of **1** (1.0 mg) or **2** (1.0 mg) in ethyl acetate (1.0 mL) was treated with 5% palladium on carbon under a stream of hydrogen gas for 16 h. Filtration of the reaction mixtures and concentration of the filtrates gave crude hydrogenated compounds. The hydrogenated products were treated with *N,O-bis*(trimethylsilyl)acetamide (0.1 mL) at room temperature for 5 min. The reaction mixtures were analyzed by GC/MS. The conditions for GC/MS measurements were as follows: Gas chromatography: column, Silicone DB1 (0.25 mm i.d. × 30 m) (J&W Scientific, Folsom, CA); injection temperature, 250°C; column temperature, 160–300°C, 3°C/min; carrier gas, He, 0.9 mL/min. Mass spectrometry: EI mode: ionization voltage, 70 eV; ionization current, 100 μA; ion source temperature, 240°C; CI mode: reactant gas, ammonia; ionization voltage, 180 eV. GC/MS spectrum of the TMS ether of the hexahydro derivative of **1**: R_t 23.2 min; CI mode: 404 ($M^+ + NH_4$, 15), 387 ($M^+ + H$, 41), 297 (100); EI mode: 259 ($M^+ - C_9H_{19}$, 100), 229 ($M^+ - C_9H_{17}O_2$, 94).

GC/MS spectrum of the TMS ether of the tetrahydro derivative of **2**: R_t 23.1 min; CI mode: 404 ($M^+ + NH_4$, 26), 387 ($M^+ + H$, 58), 297 (100); EI mode: 259 ($M^+ - C_9H_{19}$, 100), 229 ($M^+ - C_9H_{17}O_2$, 90).

Benzoylation of 1 and 2. A solution of **1** (1.0 mg) in dry pyridine (0.1 mL) was treated with benzoyl chloride (11.9 μL). The reaction mixture was stirred at room temperature for 20 min. The reaction mixture was poured into H_2O and extracted with EtOAc. The EtOAc extract was successively washed with 5% HCl, saturated aqueous $NaHCO_3$, and brine, and then dried over $MgSO_4$. Removal of the solvent under reduced pressure gave a product which was purified by HPLC (YMC, Kyoto, Japan; A-024; *n*-hexane/EtOAc, 97:3, vol/vol) to furnish **1a** (1.3 mg, 95%). Similarly, **2a** (1.4 mg, 92%) was prepared from **2** (1.1 mg).

1a: A colorless oil. $[\alpha]_D^{25}$ −37.0° (c = 0.16, $CHCl_3$). UV λ_{max}^{EtOH}nm (ε): 237 (16400). IR ν_{max}^{film} cm^{-1}: 1741, 1717, 1270, 1110, 985. ^1H NMR ($CDCl_3$, 270 MHz, δ): 0.97 (3H, *t*, *J* = 7.5 Hz, 18-H_3), 2.00–2.20 (2H, *m*, 17-H_2), 2.29 (2H, *t*, *J* = 7.5 Hz, 2-H_2), 2.91 (1H, *dd*, *J* = 6.9, 6.9 Hz, 14-H_2), 3.66 (3H, *s*, CO_2CH_3), 5.24–5.62 (4H, *m*, 9-H, 13-H, 15-H, 16-H), 5.71 (1H, *dd*, *J* = 7.3, 15.0 Hz, 10-H), 5.98 (1H, *dd*, *J* = 11.0, 11.0 Hz, 12-H), 6.62 (1H, *dd*, *J* = 11.0, 15.0 Hz, 11-H), 7.40–7.65 (3H, *m*), 8.02–8.18 (2H, *m*). MS *m/z* (%): 290 ($M^+ - PhCO_2H$, 33), 161 (5), 146 (16), 133 (20), 122 (33), 105 (100), 91 (38), 89 (32). High resolution MS: Found, 290.225; Calcd for $C_{19}H_{30}O_2$ ($M^+ - PhCO_2H$), 290.225.

2a: A colorless oil. $[\alpha]_D^{25}$ +17.3° (c = 0.05, $CHCl_3$). UV λ_{max}^{EtOH}nm (ε): 234 (20500). IR ν_{max}^{film} cm^{-1}: 1740, 1716, 1270, 1111, 985. ^1H NMR ($CDCl_3$, 270 MHz, δ): 0.87 (3H, *t*, *J* = 6.5 Hz, 18-H_3), 2.12–2.23 (2H, *m*, 14-H_2), 2.29 (2H, *t*, *J* = 7.5 Hz, 2-H_2), 3.66 (3H, *s*, CO_2CH_3), 5.40–5.60 (2H, *m*, 9-H, 13-H), 5.68 (1H, *dd*, *J* =7.6, 15.2 Hz, 10-H), 5.95 (1H, *dd*, *J* = 11.2, 11.2 Hz, 12-H), 6.59 (1H, *dd*, *J* = 11.2, 15.2 Hz, 11-H), 7.43 (2H, *t*, *J* =7.3 Hz), 7.55 (1H, *t*, *J* = 7.3 Hz), 8.05 (2H, *d*, *J* = 7.3 Hz). MS *m/z* (%): 292 ($M^+ - PhCO_2H$, 17), 164 (3), 149 (4), 134 (5), 122 (13), 105 (100), 92 (15), 87 (20). High resolution MS: Found, 292.241; Calcd for $C_{19}H_{32}O_2$ ($M^+ - PhCO_2H$), 292.240.

Preparation of 1b and 2b. (+)-α-Methoxy-α-trifluoromethylphenylacetyl chloride was added to a solution of **1** (1.0 mg), triethylamine (4.0 μL), and dimethylaminopyridine (7.6 mg) in dry CH_2Cl_2 (1.0 mL) at room temperature. The reaction mixture was stirred for 30 min, poured into water, and extracted with EtOAc. The organic layer was successively washed with 5% HCl, saturated aqueous $NaHCO_3$, and brine, and then dried over $MgSO_4$. Removal of the solvent under reduced pressure and column chromatography (*n*-hexane/EtOAc, 15:1, vol/vol) furnished **1b** (1.6 mg, 95%). In a similar manner, **2b** (1.8 mg, 96%) was prepared from **2** (1.1 mg).

1b: A colorless oil. $[\alpha]_D^{25}$ +10.2° (c = 0.11, $CHCl_3$). UV λ_{max}^{EtOH}nm (ε): 237 (20400). IR ν_{max}^{film} cm^{-1}: 1743, 1255, 1169, 989. ^1H NMR ($CDCl_3$, 400 MHz, δ): 0.97 (3H, *t*, *J* = 7.5 Hz, 18-H_3), 2.05 (2H, *dq*, *J* = 1.1, 7.5 Hz, 17-H_2), 2.29 (2H, *t*, *J* = 7.5 Hz, 2-H_2), 2.91 (2H, *dd*, *J* = 7.3, 7.3 Hz, 14-H_2), 3.54 (3H, *s*, OCH_3), 3.67 (3H, *s*, CO_2CH_3), 5.30 (1H, *ddt*, *J* = 1.5, 7.1, 10.6 Hz, 16-H), 5.38–5.56 (3H, *m*, 9-H, 13-H, 15-H), 5.63 (1H, *dd*, *J* = 7.7, 15.0 Hz, 10-H), 5.96 (1H, *dd*, *J* =11.1, 11.1, 12-H), 6.64 (1H, *dd*, *J* = 11.1, 15.0 Hz, 11-H), 7.35–7.41 (3H, *m*), 7.49–7.54 (2H, *m*). MS *m/z* (%): 290 ($M^+ - C_{10}H_9F_3O_3$, 66), 259 (6), 189 (100), 147 (25), 133 (33), 119 (32), 105 (60), 91 (63), 79 (46). High resolution MS: Found, 290.225; Calcd for $C_{19}H_{30}O_2$ ($M^+ - C_{10}H_9F_3O_3$), 290.225.

2b: A colorless oil. $[\alpha]_D^{25}$ +6.5° (c = 0.10, $CHCl_3$). UV λ_{max}^{EtOH}nm (ε): 236 (7100). IR ν_{max}^{film} cm^{-1}: 1743, 1255, 1169, 989. ^1H NMR ($CDCl_3$, 270 MHz, δ): 0.88 (3H, *t*, *J* = 6.8 Hz, 18-H_3), 2.16 (2H, *dt*, *J* = 7.3, 7.3 Hz, 14-H_2), 2.28 (2H, *t*, *J* = 7.5 Hz, 2-H_2), 3.55 (3H, *s*, OCH_3), 3.66 (3H, *s*, CO_2CH_3), 5.47–5.66 (3H, *m*, 9-H, 10-H, 13-H), 5.95 (1H, *dd*, *J* = 11.2, 11.2 Hz, 12-H), 6.61 (1H, *dd*, *J* = 11.2, 15.0 Hz, 11-H), 7.34–7.42 (3H, *m*), 7.46–7.56 (2H, *m*). MS *m/z* (%): 292 ($M^+ - C_{10}H_9F_3O_3$, 57), 261 (9), 189 (100), 105 (36), 93 (33), 81 (41), 67 (47). High resolution MS: Found, 292.240; Calcd for $C_{19}H_{32}O_2$ ($M^+ - C_{10}H_9F_3O_3$), 292.240.

RESULTS AND DISCUSSION

The $CHCl_3$/MeOH (1:1, vol/vol) extract was subjected to silica gel column chromatography which yielded a fraction containing the oxygenated fatty acids. Since the fraction was too complex to allow separation of the two fatty acids, they were isolated and characterized after being derivatized to their methyl esters. Final separation of the methyl esters was achieved by silica gel column chromatography followed by normal phase HPLC. Compound **1**, $[\alpha]_D^{23}$ −10.8°, was found to possess the molecular formula $C_{19}H_{32}O_3$ based on electron impact mass spectrometry (EI-MS) and high resolution electron impact mass spectrometry (HR-EIMS). The IR spectrum of **1** showed absorption bands due to a hydroxyl and an ester carbonyl group, while the UV spectrum exhibited an absorption maximum at 235 nm indicative of the presence of a conjugated diene. The ^1H NMR spectrum showed the signals due to a methine proton adjacent to both a hydroxyl group and an olefinic carbon along with a terminal methyl and six olefinic proton signals. Homonuclear decoupling experiments revealed the presence of the 1-hydroxy-2*E*,4*Z*-diene moiety (9-hydroxy-10*E*,12*Z*-diene in **1**) (see Experimental Procedures). They also showed that the other two olefinic protons were coupled to each other with a *J* value of 10.6 Hz indicating the presence of a *Z*-geometric olefin. The coupling pattern (15-H in **1**) of the olefinic proton signal also changed from *dtt* into *dt*

(J = 1.5, 10.6 Hz) upon irradiation of the methylene proton linked to the 1-hydroxy-2*E*,4*Z*-diene moiety. The partial structure of a 1-hydroxy-2*E*,4*Z*,7*Z*-triene was thus established. The hydroxyl group was localized by GC/MS upon hydrogenation and trimethylsilylation: GC/MS showed a fragmentation ion at *m/z* 229 and at 259 indicative of a trimethylsilyloxy group at C-9 of a C_{18} fatty acid (4). Based on these data the structure of **1** was identified as (10*E*,12*Z*,15*Z*)-9-hydroxyoctadecatrienoate. The absolute configuration at C-9 was determined by measuring the CD spectrum of the benzoate (**1a**). The CD spectrum displayed a negative Cotton curve; thus the R-configuration was assigned to C-9 (5,6). The optical purity of **1** was confirmed by the ^{1}H NMR spectrum of the corresponding (+)-α-methoxy-α-trifluoromethylphenylacetyl ester (**1b**). The ^{1}H NMR spectrum showed no signals due to its diastereomer (7). Consequently, compound **1** was concluded to be methyl (9*R*,10*E*,12*Z*,15*Z*)-9-hydroxyoctadecatrienoate.

Compound **2** $[\alpha]_D^{23}$ −5.8°, $C_{19}H_{34}O_3$, had almost the same physicochemical properties as **1** except for the molecular ion peak in MS and the number of olefinic protons in ^{1}H NMR. This suggested that **2** was a dihydro analogue of **1**. The chemical structure of **2** was determined to be methyl (9*R*,10*E*,12*Z*)-9-hydroxyoctadecadienoate.

Most lipoxygenases from various organisms have been shown to give predominantly 13*S*- or 9*S*-hydroxy acids from unsaturated C_{18} fatty acids (8). Therefore, the occurrence of 9*R*-hydroxy acids in the cyanobacterium may suggest that this organism contains an unusual type of lipoxygenase which favors 9*R*-hydroxy fatty acid formation (9). Alternatively, the oxygenated acids could be generated in the course of a biogenetic pathway involving other enzymes.

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New Phospholipid Fatty Acids from the Marine Sponge *Cinachyrella alloclada* Uliczka

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The fatty acid composition of phospholipids from the Senegalese sponge *Cinachyrella alloclada* was examined. Two new fatty acids not hitherto found in nature, namely 10,13-octadecadienoic acid and 16-tricosenoic acid, were identified. 8-Hexadecenoic, 13-nonadecenoic and 5,9,13-trimethyltetradecanoic fatty acids were also found for the first time in sponges. The latter compound (1.4% of the total fatty acid mixture), an isoprenoid fatty acid, accompanies the major fatty acid, 4,8,12-trimethyltridecanoic acid (19.7%). The monomethyl branched fatty acids (22%) identified include 23-methylpentacosanoic acid (anteiso-26:0), not previously observed in sponges. The major long-chain fatty acids encountered were the known 17-tetracosenoic, 19-hexacosenoic, 25-methyl-5,9-hexacosadienoic, 26-methyl-5,9-heptacosadienoic and 5,9,23-tricontatrienoic acids. Some sixty fatty acids were identified as methyl esters and *N*-acyl pyrrolidides by gas chromatography and gas chromatography/mass spectrometry.
***Lipids* 27, 779–784 (1992).**

Marine sponges are a source of many unusual phospholipid fatty acids (1–4). A number of sponges from different locations and orders have been investigated. Unusual structural patterns of unsaturation, methyl branching and 2-oxo-substitution have been found. The Senegalese demosponge *Higginsia tethyoïdes* (subclass *Tetractinomorpha*, order *Axinellida*, family *Desmoxydae*) has been shown to contain a series of closely related 2-methoxy fatty acids (5).

The present work is devoted to the demosponge *Cinachyrella alloclada*, formerly named *Cinachyra alloclada* before the recent taxonomic reevaluation (6) of the family *Tetillidae* (subclass *Tetractinomorpha*, order *Spirophorida*). This sponge is abundant in the same area that *H. tethyoïdes* is found, and only a few chemical studies have been published so far on *Tetillidae* sponges (7). Antimicrobial and other biological activies of *Cinachyra alloclada* have been reported (8,9). Chemotaxonomic investigations have been carried out to compare fatty acid compositions of sponges including *Cinachyra* n.sp. (10). In this latter work, fatty acids from total lipids were analyzed and classified according to chain length, degree of unsaturation and methyl branching. However no precise molecular structures were presented.

In this paper we would like to report the phospholipid fatty acid composition of the sponge *C. alloclada* collected at the Senegalese coast. Saturated, monounsaturated and polyunsaturated fatty acids, respectively, accounted for 70, 18 and 12% of the total fatty acids. Sponge specimens have been shown to contain two hitherto not described fatty acids, namely 10,13-octadecadienoic acid and 16-tricosenoic acid. Four of the sixty-one fatty acids have not previously been reported in any marine sponge, namely 8-hexadecenoic acid, 5,9,13-trimethyltetradecanoic acid, 13-nonadecenoic acid and 23-methyl-pentacosanoic acid (*anteiso*-26:0).

EXPERIMENTAL PROCEDURES

Specimens of *Cinachyrella alloclada* Uliczka (11) (Demosponge, *Tetractinomorpha, Spirophorida, Tetillidae*) were collected near Joal (~100 km south of Dakar, Senegal) at the seashore at low tide, in November 1989. These sponges, 3–8 cm in diameter, are yellow and globular. Their surface is rough, and the inhalant openings are located in aquiferous pits (porocalices). The principal skeleton is radial and composed of siliceous spicules, principally oxeas, protriaenes and spinispires. There is no differentiation of a cortical skeleton.

The sponges were washed in seawater, carefully cleaned, cut into small pieces, ground in a Waring blender with chloroform/methanol (1:1, vol/vol) and steeped twice in this solvent for 24 h (room temperature). The combined extracts yielded the crude total lipids. Phospholipids were separated from other lipids by column chromatography on silica gel (70–230 mesh) with hexane, chloroform, acetone and methanol (phospholipids) as successive eluents. For example, 170 mg of phospholipids were recovered from 220 g of sponge (dry weight).

The phospholipid fatty acids were converted to methyl esters by reaction (30 min under reflux) with methanolic hydrogen chloride, dissolved in hexane (12) and purified by column chromatography (silica gel, hexane/diethyl ether, 10:1, vol/vol). The resulting methyl esters were analyzed by gas-liquid chromatography (GLC) using a Carlo-Erba 4130 chromatograph (Milano, Italy) and a nonpolar OV-1 silica capillary column (25 m × 0.32 mm i.d. 0.40 μ); the carrier gas was hydrogen (0.5 bar, split 5/100). Thin-layer chromatography (TLC) was performed on 200 μ precoated layers of silica gel and fractions were made visible by exposure to iodine or by clearing after spraying with sulfuric acid (50%). The phospholipid composition was determined by TLC (chloroform/methanol/water, 65:25:4, vol/vol/vol). Standard phospholipid samples, and normal odd- and even-numbered fatty acid (from C_{20} to C_{31}) and monoenoic (C_{16}, C_{18}, C_{20}) fatty acid methyl esters were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Acyl pyrrolidides were prepared by direct treatment of methyl esters with pyrrolidine/acetic acid (10:1, vol/vol) under reflux (2 h) and purified by TLC on 0.5-mm layers of silica gel with hexane/diethyl ether (1:2, vol/vol) as developing solvent. Fatty acid methyl esters were hydro-

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Abbreviations: ECL, equivalent chain length; FAME, fatty acid methyl ester; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; IR, infrared; LCFA, long-chain fatty acid; MS, mass spectrum; TLC, thin-layer chromatography; 4,8,12-TMTD, 4,8,12-trimethyltridecanoic acid; 5,9,13-TMTD, 5,9,13-trimethyltetradecanoic acid.

genated by stirring (4 h), at ambient pressure and temperature, in methanol with catalytic amounts of platinum (IV) oxide (Adam's catalyst). Infrared (IR) spectra were recorded on a Perkin-Elmer model 257 spectrophotometer on liquid films (0.025 mm) between KBr plates.

In capillary gas chromatography, the temperature was programmed at 3°C/min, from 180 to 300°C for the methyl esters and from 230 to 310°C for the pyrrolidides. An initial isothermal step at 195°C for separation of short-chain fatty acids was followed by a 5°C/min gradient up to 300°C. Combined gas chromatography/mass spectrometry (GC/MS) was done on a Girdel 30 gas chromatograph linked to a Ribermag R-10-10B mass spectrometer and coupled with a Sidar computer. The GC column was a 0.32 mm × 25 m fused silica capillary column coated with OVI (0.15 μ phase thickness). The column temperature was programmed from 170 to 300°C at 3°C/min for fatty acid methyl esters and from 200 to 310°C at 3°C/min for pyrrolidides (carrier gas, helium; ion source, 220°C; ionizing voltage, 70 eV).

RESULTS

The major phospholipid classes were shown to be phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine and phosphatidylinositol. The phospholipid fatty acid composition of *C. alloclada* is shown in Table 1. As can be seen, approximately 70% of the fatty acids were saturated, of which 50% were branched ranging from 12 to 20 carbons in length. The two main phospholipid fatty acids were the isoprenoic 4,8,12-trimethyltridecanoic acid (19.7%) and palmitic acid (16:0, 8.9%); all other fatty acids were found at levels of less than 5%.

Many of the fatty acids compiled in Table 1 have been identified by comparing their equivalent chain lengths (ECL) (as methyl esters) with those of known standards, and by co-injection with standards including the LCFA methyl esters 24:0, 26:0, 28:0 and 30:0. Capillary GC analysis of a hydrogenated aliquot also provided useful information for identification of unsaturated fatty acids. For many of the components GC/MS data for the pyrrolidide derivatives were obtained to confirm the structures and to determine double bond positions. The IR spectrum of the phospholipid fatty acid methyl ester fraction displayed no prominent absorption 968–980 cm^{-1}, indicating the absence of (*E*)-unsaturation. Mass spectral data of key fatty acids are as follows:

8-Hexadecenoic acid pyrrolidide. MS *m/z* (rel. intensity), 307 (M^+, 7.8), 278(1.8), 264(2.1), 250(3), 236(5.7), 223(1.4), 222(2.0), 210(1.5), 208(2.2), 196(2.0), 194(3.2), 183(2.0), 182(9.2), 180(2.0), 168(4.0), 166(2.0), 154(3.0), 140(9.0), 127(12), 126(60.2), 113 (100), 98(33.5), 85(14), 82(7), 72(36.3), 71(20), 70(45.8), 67(13.8), 55(88).

5,9,13-Trimethyltetradecanoic acid pyrrolidide. MS *m/z* (rel. intensity), 323 (M^+, 1.4), 308(2.2), 280(1.5), 266(0.5), 252(1.2), 238(0.9), 210(2.5), 197(0.5), 196(0.8), 182(3.4), 168(3.5), 155(0.6), 154(0.9), 140(7.3), 126(16.8), 114(12.5), 113(100), 98(12.7), 85(8.5), 83(2.3), 72(13), 71(19.3), 70(17.3), 67(3.3), 57(12.3), 55(20.8).

10,13-Octadecadienoic acid pyrrolidide. MS *m/z* (rel. intensity), 333 (M^+, 7.6), 294(0.9), 290(0.8), 276(1.0), 266(0.7), 262(1.0), 250(0.8), 237(1.3), 236(2.2), 234(1.1), 224(1.7), 222(3.2), 220(1.4), 210(1.3), 209(1.0), 199(0.9), 197(0.9), 196(1.8), 194(1.6), 182(4.5), 180(1.8), 169(2.0), 168(4.7), 166(1.2), 155(1.9), 154(2.7), 152(1.3), 145(1.4), 143(2.0), 141(2.0), 140(4.9), 127(9.7), 126(37.7), 114(13.3), 113(100), 98(19), 85(9.7), 72(30.3), 70(33), 67(13.8), 57(12.7), 55(35).

13-Nonadecenoic acid pyrolidide. MS *m/z* (rel. intensity), 349 (M^+, 6.6), 334(1.5), 333(1.7), 320(1.6), 306(2.5), 294(1.5), 292(2.7), 280(4.4), 278(6.1), 266(3.8), 264(4.6), 252(3.0), 250(2.0), 239(0.8), 238(2.1), 236(1.5), 224(1.5), 211(1.2), 210(2.3), 196(3.4), 183(2.7), 182(3.7), 180(1.1), 168(4.5), 154(2.5), 140(5.0), 127(11.2), 126(42.4), 113(100),

TABLE 1

Phospholipid Fatty Acids from *Cinachyrella alloclada*[a]

Fatty acids	ECL (FAME)	Abundance in weight (%)
10-Methyl-undecanoic (*i*-12:0)	11.64	1.2
Dodecanoic (12:0)	12.00	1.0
Tridecanoic (13:0)	13.00	0.6
Tetradecanoic (14:0)	14.00	4.7
4,8,12-Trimethyl-tridecanoic (16:0)	14.52	19.7
13-Methyl-tetradecanoic (*i*-15:0)	14.66	1.9
12-Methyl-tetradecanoic (*a*-15:0)	14.73	0.7
Pentadecanoic (15:0)	15.00	1.5
5,9,13-Trimethyl-tetradecanoic[b] (17:0)	15.40	1.4
14-Methyl-pentadecanoic (*i*-16:0)	15.65	1.3
13-Methyl-pentadecanoic (*a*-16:0)	15.73	0.8
8-Hexadecenoic (16:1)[b]	15.75	1.1
9-Hexadecenoic (16:1)	15.82	0.6
Hexadecanoic (16:0)	16.00	8.9
10-Methyl-hexadecanoic (17:0)	16.34	0.8
15-Methyl-hexadecanoic (*i*-17:0)	16.65	2.3
14-Methyl-hexadecanoic (*a*-17:0)	16.74	1.3
Heptadecanoic (17:0)	17.00	0.9
10,13-Octadecadienoic (18:2)[c]	17.52	0.9
16-Methyl-heptadecanoic (*i*-18:0)	17.62	1.0
15-Methyl-heptadecanoic (*a*-18:0)	17.73	0.9
9-Octadecenoic (18:1)	17.75	2.0
11-Octadecenoic (18:1)	17.78	3.3
Octadecanoic (18:0)	18.00	4.8
17-Methyl-octadecanoic (*i*-19:0)	18.66	0.8
16-Methyl-octadecanoic (*a*-19:0)	18.74	0.7
13-Nonadecenoic (19:1)[b]	18.84	2.6
Nonadecanoic (19:0)	19.00	1.2
17-Methyl-nonadecanoic (*a*-20:0)	19.74	0.7
Icosanoic (20:0)	20.00	0.7
Heneicosanoic (21:0)	21.00	0.6
Docosanoic (22:0)	22.00	0.7
16-Tricosenoic (23.1)[c]	22.80	1.1
Tricosanoic (23:0)	23.00	0.7
17-Tetracosenoic (24:1)	23.79	3.6
Tetracosanoic (24:0)	24.00	0.8
Pentacosanoic (25:0)	25.02	1.4
5,9-Hexacosadienoic (26:2)	25.56	0.9
23-Methyl-pentacosanoic (*a*-26:0)[b]	25.74	1.1
19-Hexacosenoic (26:1)	25.83	2.4
Hexacosanoic (26:0)	26.00	0.7
25-Methyl-5,9-Hexacosadienoic (*i*-27:2)	26.20	3.1
5,9-Heptacosadienoic (27:2)	26.55	0.8
24-Methyl-Hexadecanoic (*a*-27:0)	26.73	0.6
Heptacosanoic (27:0)	27.00	0.9
26-Methyl-5,9-heptacosadienoic (*i*-28:2)	27.31	2.2
5,9-Octacosadienoic (28:2)	27.54	0.9
Octacosanoic (28:0)	28.00	0.6
5,9,23-Tricontatrienoic (30:3)	29.38	2.8
Tricontanoic (30:0)	30.00	0.7

[a]Identified minor (<0.6%) acids: br-14:0 (13.48), Δ10-17:1 (16.80), Δ11-19:1 (18.78), Δ5,8,11,14-20:4 (19.26), *a*-22:0 (21.73), 22:1 (21.88), *a*-23:0 (22.72), *i*-25:0 (24.67), 25:1 (24.90), 28.1 (27.90), 30:2 (29.53).
[b]Not previously found in sponges.
[c]Not previously found in nature.

98(17.5), 85(8.9), 83(5.6), 81(6.3), 72(23.5), 71(19.2), 70(26.7), 69(15.7), 67(8.6), 57(10.2), 55(38.9).

16-Tricosenoic acid pyrrolidide. MS *m/z* (rel. intensity), 405 (M^+, 1.4), 376(1.9), 363(4.9), 362(3.0), 348(1.2), 335 (1.6), 334(2.2), 321(2.2), 320(2.9), 306(2.7), 294(2.6), 282(2.0), 281(3.7), 280(4.0), 279(2.0), 266(2.5), 252(2.4), 238(2.2), 225(2.4), 224(2.9), 210(2.5), 196(2.4), 183(2.8), 182(3.3), 168(4.1), 155(37), 154(7.0), 141(55.3), 126(29.1), 113(94.4), 98(28.3), 97(33), 95(35.6), 85(23.6), 83(36.4), 82(18), 81(47), 79(15.5), 75(10.8), 71(100), 67(47.4), 57(58.3), 55(93).

Saturated fatty acids. The total monomethyl branched fatty acids accounted for about 22% of the total fatty acids. *Anteiso*-26:0 (1.1%) and *anteiso*-27:0 (0.6%) were identified as fatty acid methyl esters (FAME) by their ECL values of 25.74 and 26.73, respectively, and by their fractional chain lengths of 0.72–0.74 (13). The two acids remained unchanged upon catalytic hydrogenation. *Anteiso*-26:0 fatty acid has, to our knowledge, not yet been found in sponges, but is well known as a hydrogenation product of the corresponding 5,9-diunsaturated fatty acids (13,14). The *anteiso*-27:0 acid was discovered very recently in the sponge *Petrosia pellasarca* (4).

Of particular interest was the presence of a rare isoprenoic acid, not previously found in any sponge, namely 5,9,13-trimethyltetradecanoic acid (5,9,13-TMTD), which occurs together with the well known 4,8,12-trimethyltridecanoic acid (4,8,12-TMTD) (1.4% and 19.7%, respectively). The latter (4,8,12-TMTD) was readily identified by coupled capillary GC/MS of its methyl ester (5) and pyrrolidide (14) and by comparison with an authentic sample. The former (5,9,13-TMTD) showed a very low ECL value of 15.40 (as FAME) with a molecular peak at *m/z* 284 corresponding to a 17-carbon acid. The compound remained unchanged upon catalytic hydrogenation, which indicated that no unsaturation was present. These results clearly point towards methyl branching as observed previously (16). The mass spectrum (MS) of the methyl ester (base peak *m/z* 74) showed key fragments at *m/z* 101 (17%) and 129 (23%) indicative of methyl branching at the C-5 position (3% for the fragment *m/z* 115). Methyl branching at the C-9 position was indicated by significant peaks at *m/z* 171 (6%) and *m/z* 199 (4%) and by the absence of an ion peak at *m/z* 185. A very prominent peak at *m/z* 241 (16%) indicated the presence of a third methyl group at the C-13 position. The structure of 5,9,13-TMTD was confirmed by the MS of the pyrrolidide. The partial MS (Fig. 1) shows a diminished peak at *m/z* 154 flanked by elevated peaks at *m/z* 140 and *m/z* 168, clearly pointing towards the methyl group at C-5; the lack of peaks at *m/z* 224 and 294 confirmed the methyl branchings at the C-9 and C-13 positions. This isoprenoic fatty acid has not been reported in marine sponges but was recently isolated from certain fish oils (16).

Monoenoic fatty acids. Three monoenoic fatty acids were identified in *C. alloclada*. The first (1.1% of the total fatty acids) showed as FAME an ECL value of 15.75 and a molecular ion at *m/z* 268, suggesting an hexadecenoic structure. The MS of the pyrrolidide (Fig. 2) showed a molecular ion at *m/z* 307 (16:1) and key fragments at *m/z* 182 and *m/z* 194 clearly indicating unsaturation between C-8 and C-9 (Δ8 unsaturation); it also provided evidence for a lack of chain branching. The rare acid Δ8-16:1 had been detected previously (17), but no mass spectrum has been published to date.

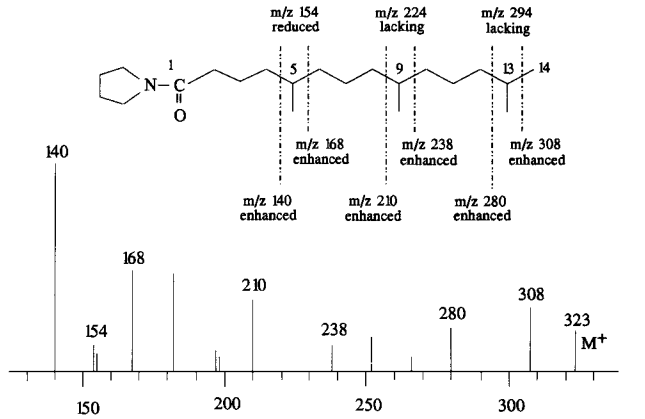

FIG. 1. Partial mass spectrum (× 10 expanded) of 5-methyl, 9-methyl, and 13-methyl-tetradecanoic acid pyrrolidide.

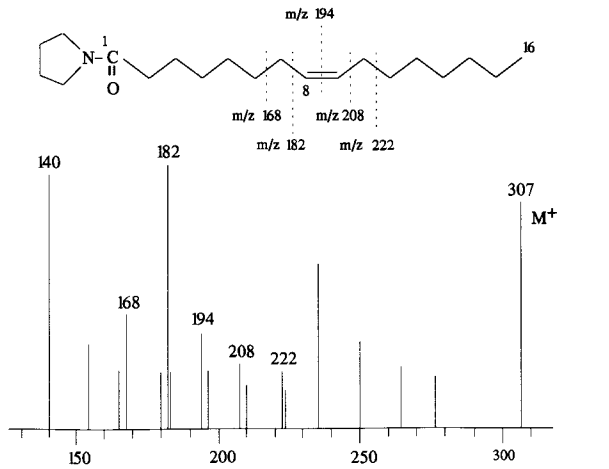

FIG. 2. Partial mass spectrum (× 10 expanded) of 8-hexadecenoic acid pyrrolidide.

The second monoenoic fatty acid (2.6%) showed as FAME an ECL value of 18.84 and a molecular ion peak at *m/z* 310 indicative of the nonadecenoic (19:1) structure. Upon catalytic hydrogenation the compound was converted to the methyl ester of nonadecanoic acid (19:0) confirming the absence of any branching. The MS of the pyrrolidide was used to locate the double bond. The spectrum (Fig. 3) showed a molecular ion peak at *m/z* 349 consistent with the nonadecenoic acid structure. A difference of 12 amu between the fragments *m/z* 252 (C-12) and *m/z* 264 (C-13) indicated Δ13 unsaturation. The data are consistent with a 13-nonadecenoic acid structure. This rather unusual fatty acid was previously found in cultures of *Thiobacillus* (18), but not in any marine sponge.

The third monoenoic fatty acid (1.1%) had an ECL value (as FAME) of 22.80 and a molecular weight of 366 amu, suggesting a 23:1 structure. Upon catalytic hydrogenation, this compound was converted to the methyl ester of tricosanoic acid (23:0), thus excluding any branching. The MS of the pyrrolidide (Fig. 4) showed a molecular ion peak at *m/z* 405 confirming the tricosenoic acid structure. A difference of 12 amu between the fragments *m/z* 294 and *m/z* 306 (C-15 and C-16) indicated Δ16 unsaturation. Therefore, we identified the acid as 16-tricosenoic acid, an n-7, odd-numbered, monoenoic, long-chain fatty acid.

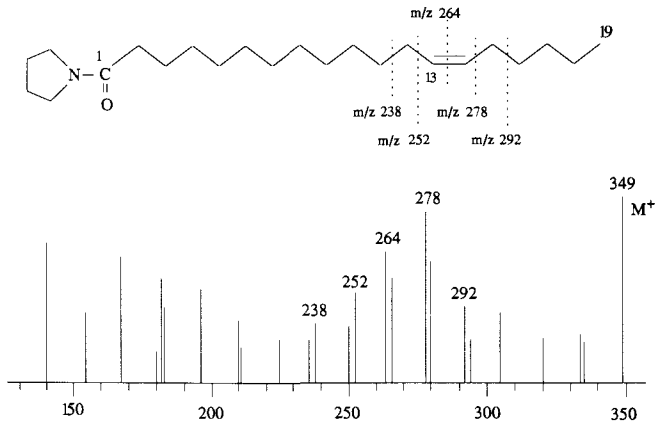

FIG. 3. Partial mass spectrum (× 10 expanded) of 13-nonadecenoic acid pyrrolidide.

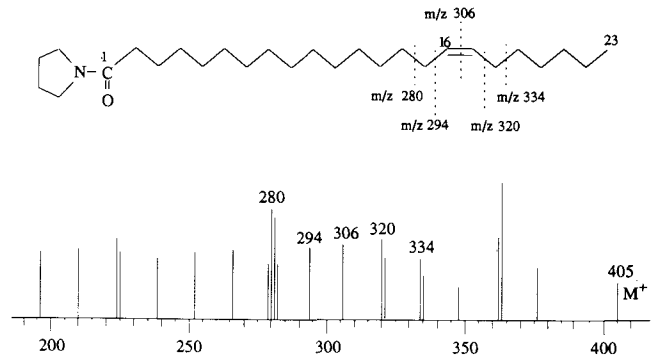

FIG. 4. Partial mass spectrum (× 10 expanded) of 16-tricosenoic acid pyrrolidide.

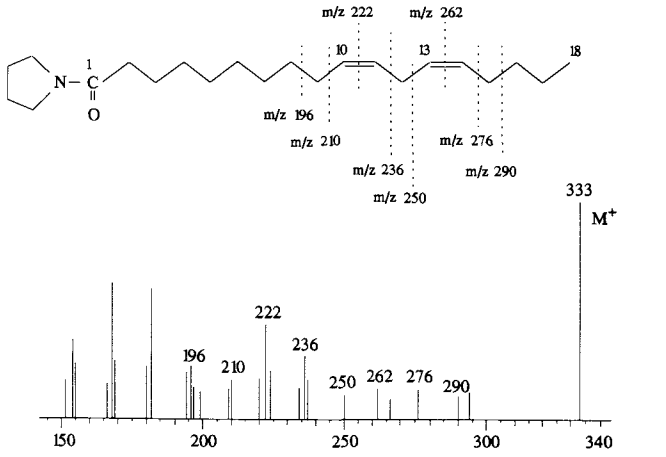

FIG. 5. Partial mass spectrum (× 10 expanded) of 10,13-octadecadienoic acid pyrrolidide.

Two other monoenoic LCFA previously found in several sponges were also identified in this study, namely 17-tetracosenoic (Δ17-24:1, 3.6%) and 19-hexacosenoic (Δ19-26:1, 2.4%) acids, which were readily characterized by their ECL values and by the MS data obtained on their pyrrolidides. The first pyrrolidide produced a molecular ion at *m/z* 419 (24:1) and showed a difference of 12 amu between the fragments *m/z* 308 (C-16) and *m/z* 320 (C-17) indicating Δ17 unsaturation. The second pyrrolidide showed a molecular ion at *m/z* 447 (26:1) and a difference of 12 amu between the fragments *m/z* 336 (C-18) and *m/z* 348 (C-19) indicating Δ19 unsaturation.

Several minor monoenoic acids were also detected and tentatively identified as 22:1, 25:1 and 28:1 acids based on their ECL values and by conversion into 22:0, 25:0 and 28:0 acids upon hydrogenation. However, the position of the double bonds could not be determined *via* the pyrrolidide derivatives due to the small amounts of material available.

Dienoic fatty acids. An interesting dienoic acid (0.9% of total fatty acids) was also detected in the phospholipid fatty acids of *C. alloclada.* Its methyl ester had an ECL value of 17.52 and a molecular ion peak at *m/z* 294 indicative of an 18:2 structure. Upon catalytic hydrogenation, the acid was converted into octadecanoic acid (18:0), excluding the possibility of any branching. The MS of the pyrrolidide (Fig. 5) showed a molecular peak at *m/z* 333 consistent with the 18:2 structure. The two double bonds were located by the 12-amu differences between the peaks at *m/z* 210 (C-9) and *m/z* 222 (C-10), indicating Δ10 unsaturation and between the peaks *m/z* 250 (C-12) and *m/z* 262 (C-13) indicating Δ13 unsaturation. The chromatographic behavior and the MS of the methyl ester have already been described (19).

Δ5,9 LCFA. Six demospongic acids possessing 5,9-unsaturation were characterized by GC (Table 1) and GC/MS (11.1% of the total fatty acids). The MS of the pyrrolidides showed the corresponding molecular peak and the key peak at *m/z* 180 (>80%) resulting from allylic cleavage (20). The third double bond of the triacontatrienoic acid could readily be located by the 12-amu difference between the peaks at *m/z* 388 (C-22) and *m/z* 400 (C-23) indicating Δ23 unsaturation. To confirm the structures, the compounds were hydrogenated and identified by coinjection with authentic straight-chain samples. The acids were 5,9-26:1; 5,9-27:2; 5,9-28:2; *iso*-5,9,-27:2; *iso*-5,9,-28:2; and the well-known 5,9,23-30:3.

DISCUSSION

Two saturated isoprenoic fatty acids, 4,8,12-trimethyltridecanoic and 5,9,13-trimethyltetradecanoic acids, were found together for the first time in a sponge. To our knowledge only the three saturated isoprenoic fatty acids have been previously isolated from sponge phospholipids: 4,8,12-TMTD, 3,7,11,15-tetramethylhexadecanoic (phytanic), and 3,7,11-trimethyldodecanoic acids (21). The monoenoic isoprenoic fatty acid 2,6,10-trimethyl-Δ5-14:1 was previously identified in *Aplysina fistularis* (20).

Our finding of a fourth saturated isoprenoic fatty acid completes a series of three homologous acids which have been encountered in sponge phospholipids to date: 3,7,11-trimethyl-12:0, 4,8,12-trimethyl-13:0 and 5,9,13-trimethyl-14:0 acids. These three acids can be derived from the phytyl side chain of chlorophyll contained in cyanobacterial symbionts (22). As suggested by Ratnayake *et al.* (16) for fish oils, a biosynthetic chain elongation of 3,7,11-trimethyl-12:0 to 5,9,13-trimethyl-14:0 may occur in sponges. 4,8,12-TMTD was identified in ten marine sponges including *Higginsia tethyoïdes* (5) and *Spheciospongia vesparium* (15) at levels of 8.1% and 23% of the

total fatty acids, respectively. This acid forms about 20% of the total fatty acids of *C. alloclada*, and samples studied at different times of the year and at different depths have all shown similar values (Barnathan, G., unpublished data). Carballeira *et al.* (23) suggested recently that 4,8,12-TMTD may be useful for taxonomic purposes in the order Hadromerida. A number of sponges do not possess this acid in their phospholipids, and we believe that this interesting suggestion should be extended to the order Spirophorida and reserved for further studies to instances in which the 4,8,12-TMTD would be found in very large amounts. However, there is little doubt that in such cases 4,8,12-TMTD is part of sponge cellular membranes as was demonstrated recently by Lawson *et al.* (24).

Ratnayake *et al.* (16) have recently identified 5,9,13-TMTD in certain fish oils, particularly in the non-urea complexing fraction. This acid, among several other isoprenoic fatty acids, has also been detected as a trace component in human milk (25) and in some shales from ancient sediments (26,27). Oxidation of 2,6,10-trimethyltetradecane by *Mycobacterium fortuitum* gave 5,9,13-TMTD as the major product (28). The association between sponges and bacteria is now well established (22,29,30) and a number of symbiotic bacteria frequently occur in massive sponges (29,31). Certain medium-chain fatty acids typically arising from bacteria have been found in sponge phospholipids (20,22), especially a number of monomethyl-branched fatty acids (*e.g.*, 10-methyl-hexadecanoic, Table 1). Significant amounts of this less common fatty acid were found in two marine sponges (14,20). The fatty acid also occurs in several bacteria, especially anaerobic sulfate-reducing bacteria, which have been observed in *Aplysina fistularis* (32).

The occurence in *C. alloclada* of the very rare monounsaturated 8-hexadecenoic acid is intriguing. It is the second case of Δ8 unsaturation found in sponge fatty acids after the case of the Caribbean sponge *Desmapsama anchorata* (Ceractinomorpha, Poecilosclerida, Myxillidae) (33). 8-Hexadecenoic acid has been described in the green alga *Chlorella kesleri* (17) but only in autotrophically grown culture. In a study (34) reported recently by Ackman (35), the body oil of menhaden fish was shown to contain 9-hexadecenoic and 8-hexadecenoic acids in a ratio of 6:1, *C. alloclada* also contains a mixture of both acids, the 8-hexadecenoic acid being predominant. The latter acid could be of dietary or symbiotic origin.

Significant amounts of 13-nonadecenoic acid were found in specimens of *C. alloclada* collected along the coast in shallow waters (Barnathan, G., unpublished results). This unusual fatty acid has been found in trace amounts in polar lipids of *Thiobacillus acidophilus* and *T. thiooxidans* (18). The Thiobacilli form a group of acid-producing aerobic microorganisms which need reduced sulfur as characteristic substrates. These marine bacteria occur primarily in shallow waters (36,37). The area of collection of *C. alloclada* is known to be rich in red algae belonging to the genus *Hypnea* (38) which contains high levels of sulfated polysaccharides (kappa-carageenan). The fermentation of thick accumulations of such algae provides reduced sulfur, and 13-nonadecenoic acid in the sponges is likely derived from a sulfur-oxidizing symbiont.

The 16-tricosenoic acid, hitherto not found in nature, completes the series of n-7 monounsaturated fatty acids found in *C. alloclada*, (Δ9-16:1, Δ10-17:1, Δ17-24:1, and Δ19-26:1 acids), all of which have been found in sponges (5,14,15,22). The monoenoic long-chain fatty acid (LCFA) can be readily explained by chain elongation from short precursors such as *cis*-vaccenic acid. The presence in *C. alloclada* of two odd-numbered n-7 monounsaturated fatty acids suggests the existence of a possible biosynthetic pathway in the odd-numbered series from 10-heptadecenoic acid, as proposed by Ayanoglu *et al.* (5). *H. tethyoides* contains several odd- and even-numbered monounsaturated LCFA which always contain the 2-methoxy group. These authors described the 2-MeO-Δ16-23:1 as a minor compound. Another interesting finding was the isolation from a Caribbean *Cinachyra alloclada* of a free long-chain glycerol monoether (17-*Z*-tetracosenyl-1-glycerol ether (7)), which could be correlated with our report of the 17-tetracosenoic acid as the major long-chain fatty acid (3.6% of the total fatty acids) in the Senegalese specimen. Work is in progress to identify other monounsaturated fatty acids found as minor components, particularly pentacosenoic and octacosenoic acids.

To the best of our knowledge, 10,13-octadecadienoic acid which we have identified here in *C. alloclada*, has not previously been found in nature. This methylene-interrupted dienoic acid has long been known as a synthetic compound (19,40). Only two methylene-interrupted octadecadienoic acids were known in sponges, Δ5,8-18:2 acid in *Microciona prolifera* (39) and the common Δ9,12-18:2 acid in *Pseudaxynissa* sp. (2). The complete series of methylene-interrupted 18:2 acids has been synthesized (19,40,41) to study the effects of double-bond position in biological systems (42), and to elucidate the structure essential to express the biological activity of linoleic acid (43,44). The Δ10,13-18:2 acid was shown to exhibit a weak specific activity for microsomal chain elongation by malonyl-CoA (45).

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Lipids and Lipophilic Pollutants in Three Species of Migratory Shorebirds and Their Food in Shepody Bay (Bay of Fundy, New Brunswick)

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To study food web transfers of lipids in the intertidal zone, three specimens of each of the semipalmated sandpiper (*Calidris pusilla* [L.]), the semipalmated plover (*Charadrius semipalmatus* Bonaparte) and the short-billed dowitcher (*Limnodromus griseus* [Gmelin]) were examined after capture during the summer of 1989. They were feeding intensively, prior to migration, on the amphipod, *Corophium volutator*, on the mudflats of Dorchester Cape, New Brunswick, Canada. The ragworm, *Nereis diversicolor*, and samples of sea foam were also collected from the mudflats at the same location in 1989 and 1990. Total body lipids of semipalmated sandpipers and short-billed dowitchers were about 20% of the wet weights, while those of the semipalmated plovers ranged from 35 to 40%. Adipose tissue fatty acids showed a trend from marine fatty acids (20:5n-3, 22:6n-3) at 10% of *C. pusilla* acids through 5% in *C. semipalmatus* to 1% in *L. griseus*. These proportions confirm differences in feeding habits proposed from analyses of gut contents. Short-billed dowitcher adipose fat included the lowest values of polychlorinated biphenyls (0.078 to 0.266 ppm). The other two species of shore-birds showed levels as high as 1.64 ppm. Concentrations of 1,1-*bis*(4-chlorophenyl)-2,2,2-trichloroethane and 2,2-*bis*(4-chlorophenyl)-1,1-dichloroethylene were also variable, but with three exceptions were ≤ 0.1 ppm of adipose fat. Sea foam collected in the tidal channels and at the edge of the water during the rising tides was found to include diverse lipophilic materials, capable of transfer to higher trophic levels. Triacylglycerols, phospholipids, sterols, hydrocarbons and pigments were typically present at a concentration of 65 × 10^3 μg/L. The long-chain isoprenoid squalene, of endogenous origin and apparently derived from feather-wax, was the only hydrocarbon present in the depot fats of the three species of birds analyzed. This indicates that the birds basically inhabited a pristine environment free of hydrocarbon pollutants. The hydrocarbon present in the sea foam consisted of a series of normal alkanes, from C_{20} to C_{39}, with no odd/even chain-length preference (carbon preference index = 1.008). These hydrocarbons probably originated in detritus from decaying vascular plants but could possibly reflect a very low level of petroleum contamination. The lack of local pollution appears to favor continued success of the migratory pattern of these birds.
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The semipalmated plover, the semipalmated sandpiper, the short-billed dowitcher and other shorebirds feed heavily on the benthic invertebrates of the mudflats around the head of the Bay of Fundy before migrating to the southern hemisphere for the winter (1–3). In building up large fat reserves, these migratory birds create a potential repository for lipophilic pollutants. If this process concentrated such materials, present at low levels in the food chain, a useful environmental marker could be created. Although the amphipod, *Corophium volutator*, is the major component of the diet of these shorebirds, its actual contribution to the diet is very different in each species. For example, *C. volutator* is, on average, 86.3% of the food of semipalmated sandpipers, but only 70% and 47.6% of the food of short-billed dowitchers and semipalmated plovers, respectively (4). The polychaetes *Nereis* sp. and *Glycera* sp., the bivalve *Macoma balthica*, and the gastropod *Hydrobia totteni* were reported (4) to be much more important in the diet of semipalmated plovers than in the diet of semipalmated sandpipers.

As a continuation of earlier studies on *C. volutator* lipids (5), we hypothesized that these dietary differences were responsible for the different organochlorine levels reported in plovers and short-billed dowitchers from the general area (6). This paper reports the lipid, hydrocarbon and organochlorine pollutant [various polychlorinated biphenyls (PCB), 1,1-*bis*(4-chlorophenyl)-2,2,2-trichloroethane (DDT) and 2,2-*bis*(4-chlorophenyl)-1,1-dichloroethylene (DDE)] contents of body fats in three shorebirds, the semipalmated plover, the semipalmated sandpiper and the short-billed dowitcher. In addition, pollutants were measured in the whole bodies of the polychaete *Nereis diversicolor*, an important constituent of the diet of shorebirds, and lipids were measured in sea foam which in turn may be an important component of the diet of benthic invertebrates.

MATERIALS AND METHODS

Semipalmated sandpipers, *Calidris pusilla* (L.), semipalmated plovers, *Charadrius semipalmatus* Bonaparte, and short-billed dowitchers, *Limnodromus griseus* (Gmelin), were captured at Dorchester Cape on the Bay of Fundy by staff of the Canadian Wildlife Service of Fredericton, New Brunswick (Table 1), and kindly supplied by Dr. P.A. Pearce. In the laboratory, adipose tissue (*ca.* 5 g) was separated from the carcasses of all birds, and the lipids of both were extracted. Ragworms, *Nereis diversicolor* O.F. Muller, were collected on November 9, 1989, at Porter Point (Minas Basin), Nova Scotia, and on May 29, 1990, at medium and upper intertidal levels near Dorchester Cape (Shepody Bay), New Brunswick. Foam samples for lipid analyses were collected at the edge of the

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Abbreviations: DDE, 2,2-*bis*(4-chlorophenyl)-1,1-dichloroethylene; DDT, 1,1-*bis*(4-chlorophenyl)-2,2,2-trichloroethane; EPA, eicosapentaenoic acid; FAME, fatty acid methyl esters; FID, flame ionization detection; GLC, gas-liquid chromatography; PCB, polychlorinated biphenyls; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography.

TABLE 1

Total Lipid Content in Whole Shorebirds (% of bird wet weight), and Contents of Hydrocarbons and Selected Chlorinated Pollutants in Their Adipose Tissue

Species with specimen number[a] and biological details[b]	Total body lipids	Hydrocarbon[c]			
		Squalene	PCB	DDT	DDE
	(% of wet/weight)	(ppm of adipose fat)			
Semipalmated sandpiper					
89128 A (8-4)	20.6	23.0	1.56	0.022	0.071
89129 J (8-4)	17.1	25.2	0.39	0.023	0.077
89131 A (8-4)	21.1	19.3	0.61	0.041	0.073
Mean	19.6	22.5	0.85	0.028	0.074
Short-billed dowitcher					
89111 A (7-14)	12.6	31.9	0.078	0.020	0.27
89112 A (7-14)	26.3	20.1	0.27	0.066	0.14
89113 A (7-14)	15.8	23.5	0.085	0.037	0.012
Mean	18.2	25.1	0.14	0.041	0.14
Semipalmated plover					
89158 A (7-8)	40.6	57.1	1.64	0.060	0.030
89161 A (7-26)	37.5	43.5	0.31	0.028	0.10
89164 A (7-26)	35.1	40.3	0.35	0.11	0.090
Mean	37.7	47.0	0.77	0.067	0.073

[a]According to Canadian Wildlife Service Tissue Bank.
[b]A, adult; J, juvenile; month-day.
[c]PCB, polychlorinated biphenyls; DDT, 1,1-*bis*(4-chlorophenyl)-2,2,2-trichloroethane; DDE, 2,2-*bis*(4-chlorophenyl)-1,1-dichloroethylene.

water during the rising tide and placed in clean glass jars, previously rinsed with clean local seawater. Foam samples were extracted with chloroform under mechanical agitation.

Total body lipids, including adipose tissue from the birds and whole ragworms, were extracted following the method described by Bligh and Dyer (7). The total body lipid content was determined gravimetrically after exhaustive removal of the solvent. A hydrocarbon-rich fraction was obtained from adipose tissue by both column chromatography using Florisil (Floridin Co., Pittsburgh, PA) (8), and by multiple thin-layer chromatography (TLC) of lipids as described elsewhere (5,9). An Iatroscan Mark III analyzer (Iatron Laboratories, Tokyo, Japan) was used for the quantitative analysis of lipid classes after separation on silica gel coated Chromarods-SII (Iatron Laboratories) (10). Squalene in the bird adipose tissue and carcasses was quantified by gas-liquid chromatography (GLC) using squalane as an internal standard. Hydrocarbon analyses were performed with a Perkin-Elmer 990 (Norwalk, CT) gas-liquid chromatograph equipped with flame ionization detection (FID), and a fused-silica wall-coated open-tubular DB-1 (J & W Scientific, Folsom, CA) column (methyl silicone; 60 m long and 0.25 mm i.d.). The preparation of fatty acid methyl esters (FAME) from other lipids and the GLC conditions are described elsewhere (11).

To examine the lipids for organochlorine contaminants, 500 mg of adipose tissue extract from birds or lipids of ragworms was digested with concentrated H_2SO_4 following the method described by Waliszewski and Szymczynski (12). Organochlorine analyses were performed on a Perkin-Elmer Sigma 4 gas chromatograph fitted with a ^{63}Ni electron capture detector and a fused silica capillary column SPB-5 (Supelco, Inc., Bellefonte, PA), 15 m in length and 0.53 mm i.d. Operating conditions were: column temperature, 240°C, carrier gas argon/methane (95:5) at a flow rate of 10.5 mL/min. PCB was quantified by comparing the detector response to four major congeners of an Aroclor 1260 standard (Supelco). For PCB analysis of *N. diversicolor* and bird tissues, extracts were cleaned up using Florisil (12). Unfortunately it was not possible to analyze foam samples for organochlorines because of insufficient sample.

RESULTS AND DISCUSSION

Hydrocarbons and biogenic lipids in shorebirds. Levels of total lipid and hydrocarbons in the three species of shorebirds collected at Dorchester Cape (New Brunswick) are given in Table 1. The total lipid contents of semipalmated sandpipers and short-billed dowitchers were 19.6% and 18.2% of wet weight, respectively. These values are similar to the lowest levels of lipid contents measured previously in five semipalmated sandpipers in this laboratory (11). The relatively low lipid contents indicate a recent arrival at the feeding grounds of Shepody Bay, where the feeding period is normally six to eight weeks (4). Lipid contents of semipalmated plovers were higher (37.7%) than in the other two species, and comparable to semipalmated sandpipers captured in the same area during the late summer of 1986 (11).

The detailed analysis of hydrocarbons present in the adipose tissue of the three species of birds showed the presence of the isoprenoid alkene squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) as a single component (Table 1). Squalene was present at 22.5 and 25.1 μg/g of adipose tissue in semipalmated sandpipers and short-billed dowitchers, respectively. Semipalmated plovers had a higher mean value, 47.0 μg/g of this lipid.

The presence of squalene as a single or dominant hydrocarbon in birds has been previously reported in the preen

(uropygial) gland and in stomach oils of several species of marine birds (13,14). Squalene is a widespread hydrocarbon among marine animals. Some species of cartilaginous fish accumulate large quantities of this hydrocarbon in their livers (14), possibly for adjusting buoyancy. Much lower levels of squalene are ubiquitous in both marine and terrestrial animals and presumably reflect the role of squalene as an intermediate metabolite for the *de novo* synthesis of cholesterol taking place mainly in the liver. The anatomical distribution of squalene and its high concentration in the feathers (11) indicate that it originated in the uropygial gland and is included in feather wax (14). Enhanced hydrophobicity of feathers is likely to be an asset in birds with this feeding pattern. The fact that no other hydrocarbon could be detected in the animals analyzed suggests that the bird population is not polluted by hydrocarbons.

Animal triacylglycerol energy reserves normally reflect the sources of food through fatty acid input. Thus, in order to obtain additional information regarding the food web structure of the mudflats of the Bay of Fundy, we analyzed the fatty acid compositions of the depot fat in the three species of shorebirds (Table 2). The proportions of polyunsaturated fatty acids typically of marine origin were distributed differently among the three shorebirds studied. Semipalmated sandpipers showed the highest level of the four n-3 polyunsaturated fatty acids (PUFA) readily available from marine invertebrates (*i.e.*, 18:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Short-billed dowitchers had the lowest proportions of marine n-3 fatty acids, and semipalmated plovers had in all cases intermediate levels between the other two bird species. The level of presumably exogenous eicosapentaenoic acid (EPA, 20:5n-3) in the bird depot fats shows perfectly this gradation from a relatively marine to a more terrestrial pattern of fatty acid composition. The EPA content in fatty acids of semipalmated sandpipers was 8.41% of the total, whereas in semipalmated plovers and short-billed dowitchers it was 4.03% and 0.87%, respectively. These fatty acid profiles are in agreement with the feeding habits of these shorebirds as described by Hicklin and Smith (4) and *C. volutator* fatty acids as reported earlier (5).

Chlorinated pollutants in shorebirds. The levels of PCB and two selected chlorinated pesticides were measured in the adipose tissue of three specimens of each species of shorebirds (Table 1). Mean values of PCB in depot fats of semipalmated sandpipers, short-billed dowitchers and semipalmated plovers were 0.85, 0.14 and 0.77 ppm of lipids, respectively. Two of these values, when converted to a wet weight basis, are very similar to those previously reported for semipalmated sandpipers and semipalmated plovers by Baril *et al.* (6). PCB levels reported here for short-billed dowitchers are, however, lower than Baril *et al.* (6) reported. Comparing the overall levels of PCB in other birds and environments (15,16), these three species of shorebirds have relatively low values.

DDT and DDE contents in sandpipers, plovers and short-billed dowitchers are shown in Table 1. Low levels of these contaminants were found in all of the shorebirds except for two semipalmated plovers, specimens No. 89161

TABLE 2

Fatty Acid Composition (w/w%) in Adipose Tissue of Three Species of Shorebirds from Dorchester Cape (New Brunswick)[a]

Fatty acid	Semipalmated sandpiper	Semipalmated plover	Short-billed dowitcher
14:0	1.67	1.95	2.57
15:0	0.24	0.43	0.94
16:0	27.60	35.58	31.81
16:1n-7	7.57	6.40	10.32
16:2n-6	0.44	0.26	0.94
16:3n-4	0.84	0.19	0.88
16:4n-1	0.51	0.21	0.07
18:0	7.60	11.00	9.24
18:1n-9	27.18	27.27	28.33
18:1n-7	2.65	2.98	4.36
18:1n-5	0.19	0.18	0.41
18:2n-6	4.51	1.05	1.87
18:3n-3	1.66	1.51	1.41
18:4n-3	0.75	0.52	0.27
20:0	0.29	0.17	0.37
20:1n-11	0.27	0.92	1.09
20:1n-9	0.43	0.63	0.33
20:1n-7	0.56	1.11	1.09
20:2n-6	0.14	0.44	0.45
20:4n-6	1.44	0.74	0.54
20:4n-3	0.14	0.22	0.87
20:5n-3	8.41	4.03	0.87
22:4n-6	0.09	0.14	0.25
22:5n-6	0.51	0.14	0.05
22:5n-3	1.15	0.98	0.36
22:6n-3	2.14	1.18	0.17
Σ Saturated	37.9	49.1	44.9
Σ Monoenoic	38.9	39.5	45.9
Σ PUFA	22.7	11.6	9.0

[a]Mean of duplicate samples.

and 89164. Mean values of DDT and DDE in semipalmated sandpipers were 0.028 and 0.074 ppm of body depot fats. Short-billed dowitchers had higher levels of DDT (0.041 ppm) and DDE residues (0.14 ppm). Pesticide residues in semipalmated plovers showed quite inconsistent results. Although the DDT levels in this shorebird were intermediate between those of the other two species, levels of DDE were low in two specimens (0.09 and 0.03), and high in a third animal (0.10 ppm). One bird (89164) was a juvenile but it did not differ as markedly in DDT burden as did one of the adults.

Chlorinated pollutants in N. diversicolor. In an attempt to investigate the possible different sources of hydrocarbons and chlorinated contaminants in this shorebird population, we measured the PCB level in the marine polychaete *N. diversicolor.* This invertebrate is an important food source for these birds while feeding on the mudflats of the Bay of Fundy. Although the amphipod *C. volutator* is the dominant component of the diet of most of the shorebirds of the area, *N. diversicolor* is also present in their diet (4).

Hydrocarbon levels in these ragworms were below detectable levels. The content of PCB in this sample of polychaetes was 0.0165 ± 0.0074 ppm of lipids. The level of chlorinated pesticides in this invertebrate in the area of study was below detectable limits. The level of PCB found in this marine polychaete is within the expected range of values considering both its environment and trophic position, and is about one order of magnitude lower than in lipids of birds, and about half of that reported in the same species inhabiting more polluted coastal areas of the North Sea (17).

Lipids in foam. In a search for lipophilic material in the Bay of Fundy, we examined lipids, fatty acids and hydrocarbons in the foam produced in the tidal channels and at the edge of the water on the mudflats of Shepody Bay at Dorchester Cape. Due to the pronounced tidal action in the Bay of Fundy, we expected a close interaction between surface water (involved in the formation of the foam) and the organic matter in the sediments. Thus the foam is considered to be a place for the concentration of organic matter present in the seawater (18,19), which would be a source of food for both filter and deposit-feeding invertebrates (20).

The lipid fraction of the foam collected in Shepody Bay was composed of an unidentified acetone-mobile component band on TLC/FID Chromarods [probably composed of algal pigments and refractory material; compare "AMPL band" described by Parrish (10) for seawater and pigments discussed by Scribe *et al.* (21)], phospholipids, sterols, triacylglycerols and hydrocarbons. The total level of lipids in the foam was 65×10^3 μg/L of foam, about 5 times that found in foam below Niagara Falls (22), suggesting that foam could be an important feature of the food web and lutant dynamics in the Bay of Fundy. The total level of hydrocarbons was, however, 750 μg/L of foam which, while very high compared to coastal bulk water concentrations (23), is just half of those found in foam below Niagara Falls (22).

An interesting feature of this lipid-rich fraction of seawater in the Bay of Fundy was the hydrocarbon composition. It consisted of a complete series of normal saturated alkanes, from C_{20} to C_{39} (and trace levels of longer chain homologous hydrocarbons), with no dominance of odd or even chain components in the overall range (carbon preference index [CPI] = 1.008) (Fig. 1). This hydrocarbon pattern is likely to reflect the input of terrestrial material or possibly low level petroleum contamination.

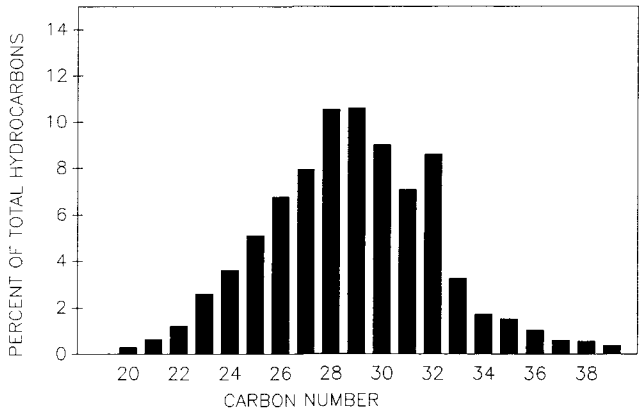

FIG. 1. Normal alkane hydrocarbon distribution in foam collected on the mudflats of the Bay of Fundy.

Information regarding composition of lipophilic material, and *n*-alkane distributions of sea water or other samples with high particle loads must be interpreted with caution (21). Both the sea water from the head of the Bay of Fundy and the foam formed from it are very turbid due to a high particle load (24). It has been reported that liquid-liquid extraction of lipids of particle-rich waters gives a low yield of high molecular weight *n*-alkanes (25). In spite of this consideration, it is interesting to point out that a very similar hydrocarbon profile was determined in the amphipod *C. volutator* in Minas Basin (5).

In order to obtain additional information regarding the origin of the organic matter present in the coastal surface seawater in Shepody Bay, we analyzed the fatty acid composition of the total lipids present in the foam (Table 3). Fatty acids in foam contained a rather low proportion of saturated fatty acids, mainly 16:0 (17.0%) and 18:0 (7.0%), since these would be expected to remain if unsaturated fatty acids were destroyed by oxidation, absorption on particles, bacterial action or other means. They were also low by comparison with the fatty acids of the shorebirds (Table 2) and seawater sampled from Nova Scotia inlets (23). The proportions of the monounsaturated fatty acids were more similar to those observed in the shorebirds, but 18:1n-9 was more than twice as abundant in foam (Table 3) than in coastal bulk water (23). Polyunsaturated fatty acids accounted for 25% of the total in foam, with almost equal proportions of fatty acids of the n-6 series (mainly 18:2n-6) and of the long-chain n-3 series. The proportions of these acids were high compared with bird adipose tissue and marine dissolved and particulate matter (23). The 18:2n-6 possibly indicates a direct terrestrial plant input, but 18:2n-6 can be common in algae as well (26).

The fatty acid profile in the foam thus indicates a heterogeneous but mostly marine origin for the organic matter input to the coastal waters in the mudflats of the Bay of Fundy. The high proportions of the labile but typical marine fatty acids, such as the C_{18}, C_{20} and C_{22} polyunsaturated fatty acids, which can be rapidly oxidized after being released into seawater, suggest a large contribution

TABLE 3

Fatty Acid Composition (wt %) of Foam Collected from Seawater at Dorchester Cape (New Brunswick)

Fatty acid	%	Fatty acid	%
14:0	4.56	20:0	0.53
15:0	1.17	20:1n-11	0.31
16:0	17.00	20:1n-9	0.50
16:1n-7	9.23	20:1n-7	0.21
16:1n-5	0.64	20:4n-6	0.79
16:2n-6	1.20	20:4n-3	0.39
16:2n-4	0.95	20:5n-3	7.33
16:4n-1	0.44	22:5n-6	0.44
18:0	7.03	22:5n-3	1.98
18:1n-9	25.74	22:6n-3	2.13
18:1n-7	2.09	Others	3.97
18:2n-6	9.89	Σ Saturated	30.3
18:3n-3	1.05	Σ Monoenoic	38.7
18:4n-3	0.43	Σ n-3	13.1
		Σ n-6	12.3

of fresh biogenic material to foam. The excess of 20:5n-3 over 22:6n-3 could indicate an origin from benthic and planktonic diatoms (26), in accordance with fatty acids of total lipids in *C. volutator* (5).

Regional environmental aspects. The values of PCB and chlorinated pesticides in the three species of shorebirds reported here were reproducible in most semipalmated sandpipers and short-billed dowitchers. Semipalmated plovers, however, showed higher and more variable levels. This is not surprising, since a recent literature review of heavy metal and chlorinated hydrocarbon concentrations in the marine environment (27) showed that variation of contamination in the same or very closely related organisms at one location can be large, often up to three orders of magnitude.

Comparable results for organochlorine pollutants have been found by Baril and co-workers (6) in semipalmated sandpipers and semipalmated plovers. The undetectable level of hydrocarbon pollution in the bird's adipose tissue is consistent with the generally uncontaminated condition of the population which migrates from presumably uncontaminated Arctic feeding grounds.

Mean concentrations of PCB in body adipose tissues in all birds were below 1 ppm. However, levels of these chlorinated pollutants varied considerably among the different species, and among individuals within species. The overall levels of chlorinated pollutants in the shorebirds analyzed and in two important local food resources (*C. volutator* and *N. diversicolor*) are confirmed as comparatively low. Although the level of PCB contamination in ragworms in the Bay of Fundy is very low compared to other areas of the world (17), PCB levels in *N. diversicolor* are higher than in *C. volutator* from the same region (5). Mean levels of DDT and DDE, and maximum individual PCB levels, in adipose tissue of semipalmated plovers, were higher than in the other two species examined. Each of the three species of shorebird has slightly different feeding habits. Semipalmated plovers, for example, include more *N. diversicolor*, and especially insects, in their diet (4). Thus, these two food items may be responsible in part for the higher levels of PCBs and pesticides found in some specimens of semipalmated plovers.

Interestingly, similar profiles of normal alkane hydrocarbons were found in both foam (Fig. 1) and in *C. volutator* (5). The source of these hydrocarbons is likely to be salt marsh plant detritus, but low-level petroleum oil contamination cannot be ruled out. While the level of PCB- and DDT-derived pollutants seems to be gradually declining in the northern hemisphere after their use was banned more than a decade ago (27), the level of petroleum contamination is likely to be rising. Therefore, any monitoring program of pollution in this region should carefully look at any possible trend in levels of hydrocarbon pollution. The information presented in this report should be useful as a reference for baseline levels.

The importance of the input of organic matter and food of terrestrial origin into the mudflats system was illustrated by the hydrocarbon composition of foam and the fatty acid composition of adipose tissue in shorebirds. In this situation we cannot exclude the possibility that chlorinated pesticides, largely used in the past on the agricultural and forest lands bordering the Bay of Fundy, are still present in the soils of certain areas, and are being very gradually released and introduced into the marine environment. Shorebirds, as far as we know, arrive with pristine fat deposits from feeding in the high Arctic. Their annual visits and systematic deposition of large reserves of fat from feeding in a small area provide natural accumulators for monitoring highly localized concentrations of such pollutants.

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Lipids in Cancer: An Introduction[1]

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It is generally believed that cancers which occur in epithelial tissues result from the interaction between host factors and the environment and that only a small percentage of these cancers can be attributed to genetic factors alone. Two of the major environmental risk factors which are under voluntary control are cigarette smoking and diet. Hence, modification of smoking and eating patterns, on a broad societal scale, offers a hopeful, feasible means of significantly reducing the incidence of an estimated 60-90% of human cancers. Among the dietary factors associated with cancer, with the possible exception of vitamin A and/or β-carotene, dietary fat has received the lion's share of attention. Although the role of dietary lipids in carcinogenesis has been known for over half a century, consideration of this topic actually began in earnest in the 1960s with the work of E. L. Wynder and of K. K. Carroll. Since then, a rapidly expanding literature, including both epidemiological, laboratory animal model and clinical studies, has appeared linking high fat intake with cancer of the breast, colon, pancreas and prostate. A look at the time trends of this phenomenon (Fig. 1) indicates an almost logarithmic increase in the number of publications over the period 1966 to 1989. It is instructive to note that the greatest attention has been paid to cancer of the breast and least to prostatic cancer. The reasons for this discrepancy are not entirely clear but appear to be related to the paucity of animal models with which to study prostate cancer and the fact that prostate cancer is more a disease of old age than breast, colon or pancreatic cancer.

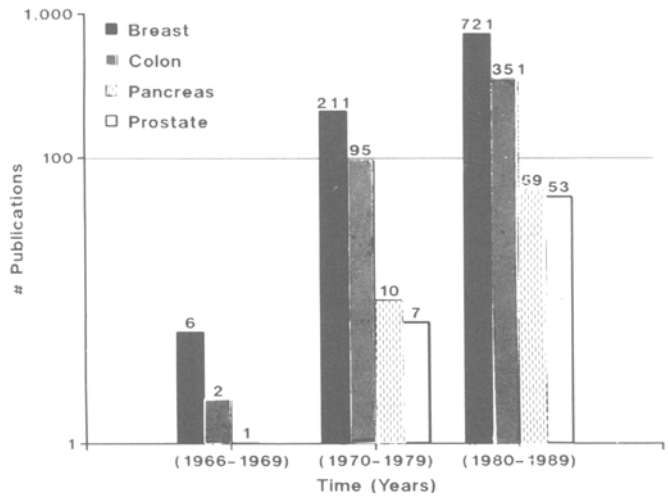

FIG. 1. Publication trends: dietary fat and cancer. Data are from Medline (National Library of Medicine) Washington, D.C.

[1]Based on a paper presented at the Symposium on Lipids in Cancer held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

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Abbreviations: EGF, epidermal growth factor; P/S, polyunsaturated/saturated fatty acid ratio; TGF_a, transforming growth factor x.

(Over 80% of clinically diagnosed prostate cancer occurs in men over age of 65.) The impact of these four epithelial cancers on the nation's health can be seen in Table 1. Taken together, they represent 40% of all cancers (incidence) and almost 30% of all cancer deaths.

The purpose of this symposium was to review critically the epidemiological, animal model, clinical and anthropological evidence for a role of lipids in each of these four cancers, to provide insight into possible mechanisms, and to discuss current thinking concerning dietary recommendations to the public for cancer prevention. Each of the contributors provided an overview of the subject at hand, ranging from the diet of paleolithic man (B. Eaton) to the current status of dietary intervention trials (N. Boyd).

The role of the type and amount of fat in breast and colon cancer was reviewed by K. K. Carroll and B. S. Reddy, respectively. By using chemically-induced mammary and colon tumor model systems, it has been shown that the effects of dietary fat are exerted primarily on the promotional phase of carcinogenesis. K. K. Carroll reviewed the evidence associating dietary fat with breast cancer, pointing out the shortcomings of retrospective case-control studies, and the need for a prospective trial involving dietary intervention. In addition, the role of different types of fat (n-3 *vs.* n-6) was addressed as was the fat-calorie controversy which, put briefly, pits the view that fat acts *via* its high caloric density *vs.* the view that fat acts independently of its caloric content.

B. S. Reddy reviewed the extensive body of experimental data linking colon cancer with high fat intake. As with breast cancer, the type as well as quantity of fat influences the development of colon tumors. High fat diets rich in triglycerides containing medium-chain (coconut oil), monounsaturated (olive oil) or n-3 polyunsaturated fatty acids (fish oil) apparently lack colon tumor promoting effects compared to oils rich in n-6 polyunsaturated fatty acids (safflower, sunflower).

The biological mechanisms underlying the fat effect in breast and colon cancer are poorly understood. Regarding breast cancer, available evidence suggests that high fat intake alters endocrine balance, eicosanoid production and/or immune functions. With regard to colon cancer, a current hypothesis holds that an increase in bile acid production, under high fat conditions, is associated with an

TABLE 1

Estimated Cancer Incidence and Mortality for Four Organ Sites: USA, 1991[a]

	Incidence	Mortality
Breast	175,000	44,500
Colon	112,000	53,000
Pancreas	28,200	25,200
Prostate	122,000	32.000
Total	437,200	154,700
Total: all sites	1,100,000	541,000

[a]Adapted from Boring *et al.* (ref. 1).

accelerated conversion by gut bacteria of primary to secondary bile acids. Secondary bile acids in turn exert colon tumor promoting activity *via* induction of polyamine synthesis and/or membrane-bound signal transduction systems.

D. P. Rose's talk provided an excellent example of the use of *in vitro* methods to unravel the effects of dietary fat at the cellular and molecular levels. After the somewhat inconsistent epidemiological evidence that dietary fat influences prostate cancer etiology was reviewed, the effect of specific types of fatty acids on the growth of human prostate cancer cells *in vitro* was described. The mechanisms underlying the stimulatory effects of n-6 and the inhibitory effects of n-3 polyunsaturated fatty acids containing fats appear to involve alterations in eicosanoid pathways. Evidence was presented suggesting that the stimulation of prostate cancer cells by n-6 polyunsaturated fats is associated with autocrine regulation by epidermal growth factor (EGF) and transforming growth factor α (TGF_α) and their respective cellular receptors. These studies, in neoplastic cells, may provide insights into the link between dietary lipids and cellular growth factors, and may provide new treatment modalities for prostate cancer patients.

Pancreatic cancer is unique in that the five year survival is only 3–4% of the cases. Hence, it is, by far, the most lethal of the lipid-responsive cancers. Like prostate cancer, studies of pancreatic cancer have lagged behind those of breast and colon largely because of the paucity of good animal models. B. Roebuck, a major contributor to this field, described the epidemiology of the disease and critically reviewed his, and other, studies which indicate that both the type and amount of fat modulate the development of chemically-induced pancreatic foci in a manner remarkably similar to that described for mammary and colon cancer. There is a need for further studies on possible mechanisms for the enhancing effect of dietary lipids on pancreatic carcinogenesis, both as a means to gain insight into etiology and to provide a rational basis for dietary intervention.

A relatively new discipline, nutritional anthropology has shown that the past weighs heavily on the present both in terms of health and disease. Based on the analysis of the dietary practices of over 20 hunter-gatherer tribes which have persisted from the late Stone Age to the present, B. Eaton and colleagues developed the intriguing hypothesis that 20th century *Homo sapiens* is maladapted to our contemporary diet. According to Eaton, contemporary man's digestive system and metabolism is structurally and functionally designed for a diet typical of the late Stone Age: high fiber (over 50 g/d), low fat (20–25% total calories) with a polyunsaturated/saturated (P/S) fatty acid ratio of substantially over 1.0. Consequently, "diseases of civilization" have resulted, at least in part, from the inability of our Stone Age metabolism to cope with diets containing 12–15 g fiber/day and 38–40% of total fat calories with a P/S ratio of approximately 0.44.

In the last analysis, however, neither animal model, nor epidemiological studies nor studies of the diets of our distant ancestors can provide proof, in themselves, that an individual's risk of cancer can be reduced by decreasing fat intake; at best, they can provide insights and clues to etiology to prove the lipid hypothesis will require a long-term prospective dietary intervention trial. Despite the weight of evidence being in favor of such a trial, all attempts at launching one in the United States have been stymied by logistic, financial and ethical considerations (2). In Canada, however, a low-fat intervention trial is underway and its progress was reviewed by N. Boyd. One of the criticisms of low-fat intervention trials has been the problem of compliance. However, both in the Canadian study and in two feasibility studies in the United States. (The Women's Health Trial [WHT] and the ongoing Women's Intervention Nutrition Study [WINS]), it has been demonstrated that free-living women can comply with a 20–25% fat diet down from 37–39% fat as calories, for months at a time. Several key issues regarding the design of such trials remain, such as the optimal balance of polyunsaturated, monounsaturated and saturated fat (the general consensus being 1:1:1), the proportion of n-6 and n-3 PUFA, and the possible interactive role of dietary fat, fiber and energy expenditure.

In conclusion, while the lipid hypothesis remains to be unambiguously proven, the overwhelming weight of evidence suggests that interim guidelines such as lowering fat intake to below 30% of total calories would serve to decrease the risk of these four cancers with few, if any, detrimental side effects. Such guidelines have the advantage that they can be amended or revised to reflect new information as it becomes available.

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REVIEW

Dietary Fat and Breast Cancer[1]

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High-fat diets are thought to increase the risk of breast cancer because animals develop mammary cancer more readily when they are fed high-fat compared to low-fat diets, and breast cancer incidence and mortality are higher in countries with high-fat as compared to those with low-fat diets. Prospective cohort studies and case-control studies have failed to provide much support for this theory, but such studies are less capable of showing the relationship because of smaller differences in dietary fat intakes of the study populations; difficulties in assessing the diets of individuals over a period of time; and possible differences in genetic susceptibility of cases and controls to breast cancer. Studies on migrants have shown that breast cancer incidence and mortality increase in populations who move from countries with low-fat to those with high-fat diets, indicating that observed geographical differences in breast cancer are due to environmental rather than genetic factors. This is supported by time-trend studies showing that breast cancer increases in countries as the level of fat in the diet rises. Controlled, long-term dietary trials are needed to determine whether the converse is true: namely, that reduction of dietary fat can reduce the risk of breast cancer. Large groups are required to achieve statistical significance, but smaller numbers may be adequate for studies on high-risk individuals. Preliminary experiments already have demonstrated the feasibility of carrying out such dietary trials.
Lipids 27, 793-797 (1992).

The idea that dietary fat has an important influence on breast cancer incidence and mortality is based primarily on the results of studies on experimental animal models and on on inter-country correlations derived from epidemiological data.

Studies on the effects of dietary fat on mammary cancer in animals began with the work of Tannenbaum (1) and were continued in a number of laboratories over a period of about 15 years (2). The experiments consistently showed that mice and rats developed mammary tumors more readily when they were fed high-fat as compared to low-fat diets, but these observations attracted relatively little attention until they were correlated with epidemiological data from different countries showing that breast cancer mortality increases with increasing levels of dietary fat (3-5).

This evidence for breast cancer and similar findings for cancer at other sites, such as the colon, led the National Research Council (NRC) Committee on Diet, Nutrition, and Cancer (6) to conclude ". . . that of all the dietary components it studied, the combined epidemiological and experimental evidence is most suggestive for a causal relationship between fat intake and the occurrence of cancer."

In contrast to the data from experimental studies and inter-country correlations, the results of case-control and prospective cohort studies have not provided strong evidence for an association between dietary fat and breast cancer (7-11). There also have been suggestions that energy balance has a more important influence on breast cancer than dietary fat (11,12).

Meanwhile, the observations implicating dietary fat in breast cancer have stimulated dietary trials which have demonstrated the feasibility of reducing the level of fat to about 20% of total calories in the diets of North American women (13,14). It is not possible to draw conclusions regarding the effect of dietary fat reduction on breast cancer based on the results to date, but other benefits have been reported, including a reduction in plasma or serum cholesterol (14,15) and relief from symptoms of cyclical mastopathy (16).

The aim of this paper is to discuss the evidence in more detail and to present ideas designed to stimulate more experimentation and discussion on the role of dietary fat in breast cancer.

DEVELOPMENTAL ASPECTS OF BREAST CANCER

In countries where breast cancer is prevalent, the incidence is much higher after menopause than before (17). However, there is substantial evidence to suggest that initiation occurs at a much younger age. Thus, early menarche increases the risk of breast cancer while an early first pregnancy decreases the risk (8,18). Such observations point to an early onset and suggest that hormonal factors play an important part in development of the disease (19,20).

Epidemiological data on migrants from countries such as Japan, China, Poland and Italy to the United States or Australia have shown that the incidence of breast cancer increases rather slowly (21). Similarly, in post-war Japan, breast cancer mortality has increased more slowly than that of colon cancer (8). These observations also suggest a relatively long latent period for breast cancer.

Experiments on animals have provided evidence that initiation of mammary cancer is more likely to occur at a young age. Carcinogens such as 7,12-dimethylbenz(a)anthracene (DMBA) and *N*-methylurea (NMU) are much more effective in young compared to older animals (22,23). Studies in our laboratory showed that using a low dose of DMBA for initiation greatly extended the latent period while still producing a high incidence of mammary cancer (24,25). This may be similar to the pattern of development of breast cancer in humans, since humans are more likely to be exposed to low rather than high doses of carcinogenic agents.

Russo *et al.* (26) have done extensive histological studies comparing the development of mammary tumors in rats and in humans. They have shown that the incidence of mammary tumors induced in rats by DMBA is directly proportional to the density of highly proliferating terminal end buds, which is highest in young animals and declines sharply after 55 days of age (27). In humans, cell replication in terminal ducts of the mammary gland reaches a peak in early adulthood and decreases considerably with age (26).

[1]Based on a paper presented at the Symposium on Lipids in Cancer held at the AOCS Annual Meeting, Baltimore, MD, April 1990.
[2]Career Investigator of the Medical Research Council of Canada.
Abbreviations: DMBA, 7,12-dimethylbenz(a)anthracene; NMU, *N*-methylurea.

These observations are compatible with the idea that initiation of breast cancer is more likely to occur in young women when the breasts are developing and the epithelial cells are dividing at a more rapid rate. The differentiation of cells associated with pregnancy and lactation appears, however, to render them less susceptible to transformation, although pregnancy ensuing after initiation can accelerate the carcinogenic process (28).

As suggested above, hormonal factors evidently play an important role in the initiation of breast cancer. This idea is reinforced by observations that the ability of carcinogens, such as DMBA and NMU, to induce mammary cancer in rats varies with the stage of the estrous cycle at which they are administered (29,30). The hormonal environment also appears to be an important factor in later stages of breast cancer development, since ovariectomized women and women who experience early menopause appear to be at lower risk (8). Environmental factors, such as diet, also may have a significant effect on breast cancer by modulating its rate of development (4,5).

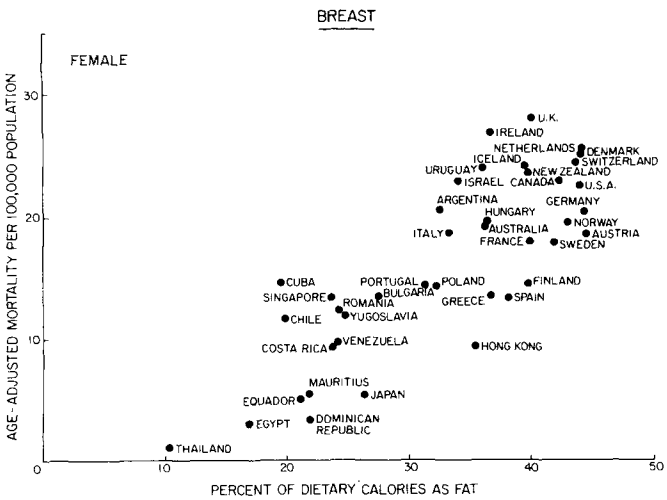

FIG. 1. Correlation between percentage of dietary calories as fat and age-adjusted mortality from breast cancer in different countries (reproduced from Carroll; ref. 38).

DIETARY FAT AND BREAST CANCER

In studies on diet in relation to breast cancer, much of the emphasis has been focused on dietary fat (6,7). This is based on epidemiological evidence showing a strong positive correlation between dietary fat and breast cancer incidence and mortality in different countries (3,31); and on laboratory experiments showing that animals fed high-fat diets develop mammary cancer more readily than those fed low-fat diets (3,5). This has been observed consistently in animals fed *ad libitum*, both with spontaneous tumors and with tumors induced by various means (32).

Studies on migrating populations have provided evidence that geographical differences in breast cancer incidence and mortality are related to environmental rather than genetic factors (21). This is supported by time trends showing marked increases in age-adjusted death rates from breast cancer in a number of countries in recent years (33,34).

In contrast to the strong positive correlation between dietary fat and breast cancer incidence and mortality observed in inter-country comparisons, case-control and prospective cohort studies have shown little evidence of such a relationship (6–8). There are, however, a number of possible reasons for this apparent discrepancy (35–37).

Case-control and prospective studies usually involve smaller and more homogeneous populations than those on which inter-country comparisons are based, and the range of dietary fat intake may not be sufficient to show marked differences in breast cancer. For example, inter-country comparisons show little correlation between breast cancer mortality and dietary fat among countries where dietary fat contributes more than 30% of total calories, but in most of those countries breast cancer mortality is higher than in countries where fat accounts for less than 30% of calories (Fig. 1). Overall, the data plotted in Figure 1 suggest the possibility of a straight-line relationship between breast cancer mortality and percent of dietary calories as fat. Breast cancer clearly causes less mortality in countries where a smaller proportion of dietary calories comes from fat, but the scatter in the data, which may be due to a variety of factors, makes it difficult to distinguish effects of small increments of dietary fat.

Another problem with case-control and prospective cohort studies is the difficulty of measuring the fat intake of individuals (35,36). Even if it can be measured with reasonable accuracy at a given point in time, the dietary habits of individuals change with time, and it is nearly impossible to assess their intake over periods of years, particularly when it involves dietary recall.

A more fundamental problem with case-control studies relates to the influence of genetic or other non-dietary factors that influence breast cancer incidence and mortality. Even in countries where breast cancer is more prevalent, only about 10% of women develop the disease, so those who do not are presumably less susceptible to environmental risk factors. Thus, the failure of control subjects to develop breast cancer on a high-fat diet may be related to this lack of susceptibility, whereas the more susceptible cases may develop the disease even when their fat intake is lower than that of the controls. In comparing relatively small numbers of cases and controls, the differing characteristics of individuals increase the difficulty of detecting effects of diet or other environmental factors. Even so, a recent analysis of a number of case-control studies indicated that cases had significantly higher fat intake than did controls (10).

Studies on migrants suggest that the range of genetic susceptibility to breast cancer is similar in the populations of different countries, because with time, the incidence in migrants approaches that of their country of adoption. In comparing large populations with similar distribution of susceptibility to breast cancer, a large proportion of each population will not develop cancer under the environmental conditions existing in either country. However, a certain proportion of the more susceptible individuals may develop it under the conditions of one country (Environment B) but not under those of the other (Environment A) (Fig. 2). It is thus possible to observe the effects on breast cancer of environmental variables, such as dietary fat, by comparing incidence or mortality in two populations having roughly similar proportions of individuals with comparable susceptibility to the disease. The hypothetical variation in genetic susceptibility to

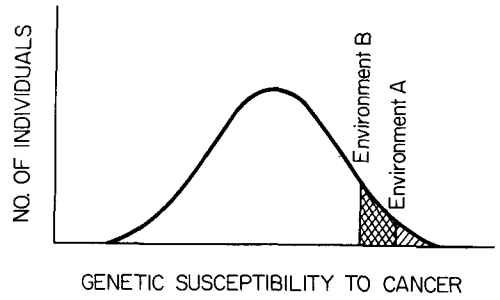

FIG. 2. Hypothetical variation in genetic susceptibility to breast cancer in a large population of women. Because of lower genetic susceptibility, most women will not develop breast cancer, even under environmental conditions that promote the disease (represented by the open area under the curve). Highly susceptible individuals will develop breast cancer under almost any set of conditions (represented by the shaded area to the right of Environment A). Individuals with a relatively high susceptibility, who may be protected from breast cancer under one set of environmental conditions, but not under another, are represented by the cross-hatched area between Environments A and B (Reproduced from ref. 39).

breast cancer shown in Figure 2 may be due partly to differences in the genomes of different individuals, and partly to variations in exposure to carcinogenic stimuli during their lifetime.

EFFECTS OF DIFFERENT TYPES OF DIETARY FAT

Experiments in animals have provided evidence that promotion of mammary cancer is influenced by both the amount and type of dietary fat (40). In particular, there is a requirement for a certain amount of polyunsaturated fat, up to a level of 4–5% of total calories (41–43). When this requirement is met, the incidence and yield of mammary tumors induced by DMBA increases in proportion to the total amount of fat in the diet (42,43). Dietary fish oil has an inhibitory effect on mammary carcinogenesis when fed at higher levels in the diet (44,45), but a relatively high proportion of fish oil to polyunsaturated vegetable oil is required to produce the effect (46).

In contrast to results obtained with experimental animals, the polyunsaturated fat content of the diet shows little correlation with breast cancer mortality in humans (47). This is probably because the polyunsaturated fat content of most human diets is above the threshold required for maximum effect on cancer promotion. Thus, the observed positive correlation between dietary fat and breast cancer mortality in humans is more likely to be related to total fat intake than type of fat.

Saturated and monounsaturated fatty acids predominate in the higher fat diets consumed in Western industrialized countries, and both tend to increase as total fat increases. This is not true for polyunsaturated fatty acids, which vary over a much narrower range and do not show a correlation with total dietary fat (48). As a result, both saturated and monounsaturated fatty acids are positively correlated with breast cancer mortality in human populations, whereas polyunsaturated fatty acids are not (47).

DIETARY FAT *VS.* CALORIC INTAKE

Because fat is a much more concentrated source of energy than either carbohydrate or protein, high-fat diets have a high energy density as compared to low-fat diets. Dietary protein varies over a narrower range than either fat or carbohydrate (49), so high-fat diets are generally low in carbohydrate and *vice versa*. The higher energy density of high-fat diets tends to encourage a higher caloric intake and, thus, there is a strong positive correlation between dietary fat and calories (5).

Many experiments have shown that carcinogenesis can be inhibited in experimental animals by dietary restriction (2,4,50), and this has been observed with high-fat as well as with low-fat diets (51). Such observations have led to the conclusion that dietary fat exerts a promoting effect on mammary carcinogenesis only under *ad libitum* feeding conditions (12,52). Although it is difficult to dissociate effects of dietary fat from those of caloric intake, Freedman *et al.* (53) recently concluded on the basis of a survey of a large number of animal experiments that higher caloric intake and higher fat intake independently increase mammary tumor incidence in rats and mice. Animals given the opportunity to exercise voluntarily were observed to have a lower incidence of mammary cancer than their sedentary counterparts (54), so energy utilization also appears to be an important factor in mammary carcinogenesis.

It seems rather impractical to suggest that people reduce their caloric intake in the presence of an abundance of food, even if it were to reduce their risk of developing cancer. They can, however, be persuaded to reduce their fat intake by eating a diet containing larger amounts of complex carbohydrates, and feasibility trials have shown that women will adhere to lower fat diets for prolonged periods of time (14,55). If the objective is to decrease the risk of breast cancer and if it can be achieved by reducing dietary fat intake, this appears to be a more practical approach than trying to restrict food intake.

RATIONALE FOR CLINICAL TRIALS ON DIETARY FAT AND BREAST CANCER

The positive correlation between breast cancer incidence and mortality and dietary fat in different countries does not necessarily indicate a causative relationship. Increasing the level of dietary fat in diets fed to experimental animals clearly increases their susceptibility to mammary cancer, but this finding cannot be extrapolated to humans with certainty. The studies on migrants and the changing trends in cancer mortality with time provide strong evidence that observed differences in breast cancer mortality are due to environmental rather than genetic factors. The populations involved in these studies have typically increased their dietary fat intake and have experienced an increase in breast cancer incidence and mortality.

It is very desirable to know whether the converse is true (*i.e.*, that reducing the level of dietary fat would lead to a decrease in breast cancer), and it appears that the only way to establish this is by conducting controlled dietary trials. The original Women's Health Trial did not proceed beyond the feasibility stage for a variety of reasons, including the expense involved. However, there is now re-

newed interest in this type of trial, which can be designed to investigate effects on other health problems, such as cardiovascular disease, and thus provide more useful information with little added expense (56).

Trials on healthy women or women with moderately increased risk of breast cancer require relatively large numbers of subjects, who need to be followed for lengthy periods of time. In the case of women with benign breast disease, who have a substantially higher risk of developing breast cancer (55,57,58), the number of subjects required to assess the effect of dietary fat is likely to be smaller. There is, however, the possibility that the response of these subjects may not be typical of that for the population as a whole.

Another study population consists of women who have already developed breast cancer. In this case, the primary aim is to determine whether reducing the level of dietary fat will decrease the risk of recurrence of cancer (59). There is already some evidence that recurrence occurs less frequently in populations on low-fat compared to those on high-fat diets (60).

CONCLUSIONS

Most dietary guidelines that have been issued in recent years have advocated a reduction in dietary fat as a means of preventing obesity and reducing the risk of a number of chronic diseases, including cancer. These guidelines are based on a substantial amount of scientific data, but definitive proof of their validity is still lacking. It therefore seems appropriate to recommend strongly that the potential benefits of low-fat diets be tested by controlled dietary trials. The cost of such trials is miniscule compared to the current economic and social burden of diet-related chronic diseases.

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Dietary Fat, Fatty Acids and Prostate Cancer[1]

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International comparisons suggest a relationship between prostate cancer incidence and dietary fat, an inference supported by migration studies, the changing incidence rates and levels of animal fat consumption in Japan and the results from some case-control studies. Overall, however, epidemiological studies have been inconclusive, and although prostate cancer is one of the hormone-dependent tumors, evidence of interactions between dietary fats and male endocrine function is incomplete. Laboratory experimentation has shown that n-6 fatty acids stimulate and n-3 fatty acids inhibit human prostate cancer cells in culture; also, feeding diets rich in marine oils suppresses growth of these cells as solid tumors in athymic nude mice. These growth effects of polyunsaturated fatty acids appear to involve both prostaglandins and leukotrienes and to interconnect with autocrine regulation by epidermal growth factor-related polypeptides.
Lipids 27, 798–803 (1992).

Prostate cancer is now the second leading cause of cancer deaths in males, and the most frequently diagnosed cancer among men in the United States (1). Moreover, because of the changing age distribution, there will inevitably be further increases in cases and prostate cancer-related deaths as time goes on.

In general, the populations with a high incidence of prostate cancer are those of northern Europe, or of northern European origin, although African-Americans, even after adjustment has been made for disparity in socioeconomic status, constitute an important exception to this general rule (2). Intermediate in rank are the southern European countries and those of Latin America, while the Far East shows the lowest mortality rates. Yet, despite the pronounced differences in clinically manifest prostate cancer, and consequently in the mortality rates, cancers which are restricted to the prostate gland and have remained undiagnosed in life ("latent" or "histological" prostate cancer) are found at autopsy with about the same frequency in Japanese men as in white males in the United States, Canada, England and Austria (2). This observation was refined by Akazaki and Stemmerman (3) who, having noted that the prostate cancer mortality rate for Japanese in Hawaii is closer to that of the American white population than to the rate for native Japanese, performed a histological study of latent carcinoma of the prostate. They found no difference in the actual prevalance rates between the two groups, but larger lesions suggestive of rapid growth rates were more common among the migrants to Hawaii.

Jackson *et al.* (4) made a pathological comparison of prostate cancers in high-risk African-Americans and low-risk black Africans. Included was autopsy material from 249 males in Ibadan, Nigeria, and 243 in Washington, D.C.; none had had clinically manifested prostate cancer. Small ("microscopic") carcinomas of the prostate were detected with approximately equal frequency in the two groups, whereas invasive cancer was more common among the African-Americans.

Another internationally based autopsy study compared the frequency and histopathological characteristics of latent prostate cancers in seven geographical areas (5). Small, so-called "focal," latent carcinomas occurred with approximately the same incidence in Hong Kong, Singapore, Israel, Jamaica, Uganda, Germany and Sweden. However, although larger, diffuse infiltrating carcinomas within the prostate gland were common in the two European and in the Jamaican black populations, they were seen much less frequently in the two Chinese and the Jewish population. From these results, it appears that diffuse latent carcinomas are very likely on their way to becoming clinically manifest, whereas focal latent prostate cancers are usually either extremely slow in their progression, or are truly in a state of growth arrest and potential regression.

Carter *et al.* (6) have proposed that while phenotypic changes have occurred in the cells that constitute what is commonly designated a "focal latent carcinoma," a term which they avoid, additional events are necessary for the emergence of invasive clinical disease. While the biological mechanisms which determine the step-wise progression of a few transformed prostatic epithelial cells to a life-threatening, clinically overt, cancer remain to be elucidated, one possibility is that dietary fat, or specific fatty acids, are involved in this process.

DIETARY FAT, OBESITY AND PROSTATE CANCER

The putative involvement of dietary fat in breast cancer etiology is discussed elsewhere in this issue. Figure 1 shows that, overall, there is a fairly strong positive correlation between the mortality rates for breast and prostate cancer in different countries. Particularly noteworthy is the low risk for both of these forms of cancer in Japan, an oriental country which shares many of the environmental hazards of the western industrialized countries. Wynder *et al.* (2) pointed out that when Japanese males migrate to the United States their risk of dying of prostate cancer increases and suggested that adopting the typical American diet, relatively high in fat, might be a contributing factor. Similar arguments do, of course, apply to breast cancer.

Armstrong and Doll (7) showed that prostate cancer mortality rates for different countries were highly correlated with estimates of total fat consumption. Using nutritional data published by the Food and Agriculture Organization of the United Nations and international cancer mortality statistics prepared by Kurihara *et al.* (8) we have confirmed and extended their observations. Figure 2 confirms that there is a strong correlation ($r = 0.704$) between available fat from animal sources for 28 countries,

[1]Based on a paper presented at the Symposium on Lipids in Cancer held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

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Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; DHA, docosahexaenoic acid; EGF, epidermal growth factor; EPA, eicosapentaenoic acid; LA, linoleic acid; LT, leukotriene(s); NMU, *N*-nitrosomethylurea; PG, prostaglandin(s); PUFA, polyunsaturated fatty acid(s); TGF-α, transforming growth factor α.

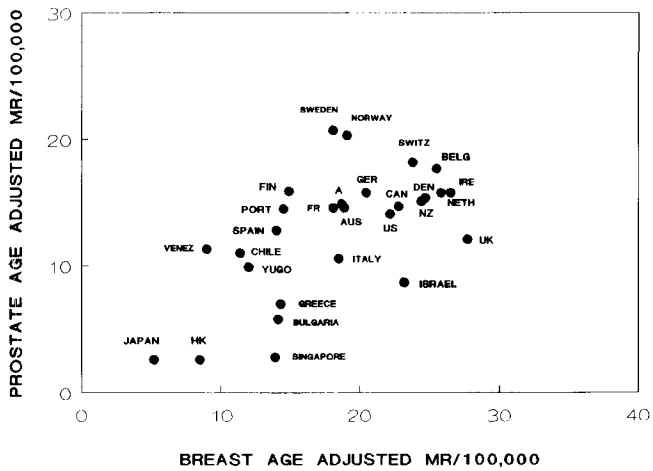

FIG. 1. The correlation between age-adjusted prostate and breast cancer mortality rates for 28 countries (r = +0.619). The U.S. data are for whites only. AUS, Australia; A, Austria; FR, France.

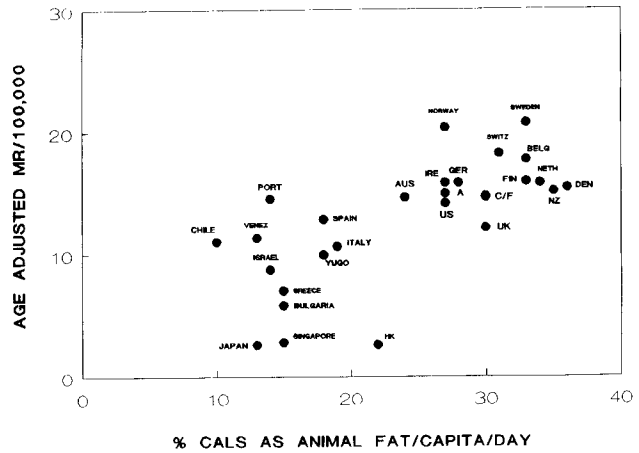

FIG. 2. The correlation between age-adjusted prostate cancer mortality rates and estimates of daily animal fat consumption for 28 countries (r = +0.704).

expressed as percent of total calories *per capita* per day, and the corresponding age-adjusted prostate cancer mortality rates. However, a relationship is completely absent when only fats from vegetable sources are examined (Fig. 3). When we sought correlations between the estimated consumption of various food groups in 30 different countries and prostate cancer mortality rates, we found a strong positive correlation with milk, a much weaker one with meats, and a strong negative relationship to the consumption of cereal products (9).

In general, there is a strong correlation between fat consumption and energy intake. However, when we examined the data for 28 countries, we found only a weak association between prostate cancer mortality rates and estimates of daily caloric intake (9). Next, the sources of energy were considered, and the results followed those observed for dietary fat; a strong positive correlation was seen when only calories of animal origin were included (r = 0.68), whereas for vegetable-derived calories the relationship was a negative one (r = −0.44).

A number of case-control studies have been performed to examine the relationships between prostate cancer and dietary fat intake. Table 1 summarizes eight of these, all but one of which provide some support for the association. Particularly convincing are the results from studies (12,13,16) which showed a positive relationship between increasing estimates of fat intake and risk. The exception is a study from Japan, which found no relationship between prostate cancer risk and the consumption of meat and milk (17). Likewise, a prospective investigation of

TABLE 1

Dietary Fat and Prostate Cancer Risk: Case-Control Studies

Study: First author (ref)	Study design	Outcome
Rotkin (10)	111 matched-pairs, hospital controls.	Association with animal fats: excess intake of pork, dairy products, eggs.
Schuman *et al.* (11)	Case control study.	Higher intake of margarine and other high-fat foods.
Graham *et al.* (12)	262 cases and 259 hospital controls, excluding those with digestive diseases. All white.	Association with animal fats: increasing intake of meats and fish. Statistically significant, and with a clear dose-response only for those ≥70 yr, similar trends for those <70 yr.
Kolonel *et al.* (13)	243 cases and 321 population controls; all 5 ethnic groups in Hawaii included.	≤60 Years, no association; ≥70 years, positive dose-response gradient for total fat; gradient for total fat.
Heshmat *et al.* (14)	Age and race-matched case-control study, 181 pairs, all black patients. Food frequency questionnaire for when aged 30–49 and ≥50 years.	Total and saturated fats higher at age 30–49 years, but not statistically significant at $P < 0.05$.
Talamini *et al.* (15)	Italian study of 166 cases and 202 hospital controls.	Significant positive relation with milk, cheese and meat consumption.
Ross *et al.* (16)	142 matched-pairs; population controls. White- and African-Americans.	Higher (animal) fat intakes associated with increasing risk: positive dose-response.
Mishina *et al.* (17)	Japanese study of 100 cases and population controls.	No association.

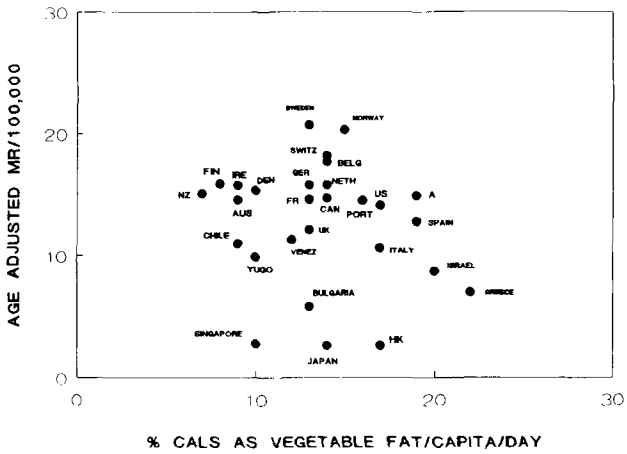

FIG. 3. The lack of correlation between age-adjusted prostate cancer mortality rates and estimates of daily vegetable fat consumption for 28 countries.

122,261 Japanese males failed to detect any association between these two fat sources and risk (18).

Despite these negative results, which could conceivably be due to the inaccuracy of dietary recall data when obtained from a low-risk, homogeneous population, most of whom are consuming low levels of the foods in question, an examination of time-trends in Japan does provide some indirect support for a relationship between dietary fat and prostate cancer risk. Wynder *et al.* (19) reevaluated this aspect of cancer epidemiology and pointed out that there has been a fourfold increase in fat consumption, notably from animal sources, in Japan since the early 1950s. While these dietary changes have occurred, prostate and breast cancer incidence rates have increased steadily since the late 1960s. But, on the other hand, when Severson *et al.* (20) studied 7,999 men of Japanese ancestry born and living in Hawaii, whose prostate cancer risk has been calculated to be 10 times that in Japan (21), dietary fat, estimated from 24-h recall data, was not found to be associated with prostate cancer.

A large prospective study of 6,763 white American Seventh-Day Adventists was performed by Snowdon *et al.* (22). They observed a significant positive association with milk consumption, and suggestive but weaker ones for dairy products and meat consumption and risk of death from prostate cancer.

Obesity has been associated with breast cancer risk in most, but not all, studies of postmenopausal women (23,24), and the same relationship has been sought in studies of prostate cancer. The results of seven reports, three of which were positive, are summarized in Table 2. Overall, it seems prudent to conclude on present evidence that obesity is unlikely to be a strong risk factor for prostate cancer, and that any positive association may be due to a third element which is common to both high body weight and prostate cancer risk.

HORMONES AND PROSTATE CANCER: RELATION TO DIETARY FAT

Both prostate and breast tissues are targets for endocrine action, and cancers arising from both of these sites possess steroid receptors and are hormone-dependent at some stage in their development. Suppression of estrogen activity is standard therapy for breast cancer, as is elimination of androgen stimulation in the case of prostate cancer.

Many studies have been performed in an attempt to demonstrate abnormal hormone levels in blood from prostate cancer patients, and, specifically, elevations in the serum androgens. No consistent differences compared with the serum levels of healthy men of similar age have been detected, perhaps because these operate in early life and are no longer evident at the time of diagnois (27).

The results of a study performed by Ross *et al.* (28) provide support for this interpretation. They compared the total and nonprotein-bound (biologically available) serum testosterone levels in college-aged African-American and white-American males. The black men, who possess the highest risk of developing prostate cancer in later life of all population groups worldwide, had significantly higher total and unbound testosterone levels. The authors considered the possibility that dietary fat intakes were involved in this difference in circulating androgens, but concluded that this explanation was unlikely because nutritional surveys in the United States have demonstrated no real differences in dietary fat intakes between blacks and

TABLE 2

Obesity and Prostate Cancer Risk

Study: First author (ref)	Study design	Outcome
Wynder *et al.* (2)	Case-control; 300 cases and 400 hospital controls.	No association.
Greenwald *et al.* (25)	Retrospective case-control; 268 fatal cases and 536 population controls.	No association between college age body weight and later risk.
Garfinkel (26)	Prospective; 336,442 American males.	Positive: mortality ratio 1.33 for those 130–139% above average weight.
Graham *et al.* (12)	Case-control; (Table 1).	No association.
Snowdon *et al.* (22)	Prospective; 6,763 Seventh-Day Adventists.	Positive: compared with 90–109% ideal body weight, relative risk of 2.5 for those 130–249%.
Talamini *et al.* (15)	Italian case-control (Table 1).	Compared with <65 kg, risk estimates 2.3 for 85–94 kg, and 3.0 for ≥95 kg.
Ross *et al.* (16)	Case-control (Table 1).	No association.

whites when matched, as was done in their study, for socioeconomic status.

Jackson *et al.* (4) included serum hormone assays in their comparative study of blacks in the United States and Nigeria, and did find that the testosterone and estradiol concentrations were lower in African black prostate cancer patients compared with the corresponding controls. Moreover, among the controls there were trends for the testosterone, but not estradiol, levels to be higher among the African-Americans. Dietary data were not included in this investigation.

There is, however, evidence that diet can influence blood hormone levels in men. Obesity is accompanied by *reduced* plasma testosterone concentrations (29,30); the percentage that is nonprotein-bound shows little or no change (30), but because the plasma sex hormone-binding globulin concentration in middle-aged men is negatively correlated with body weight (31), the absolute level of biologically active testosterone is not necessarily reflected in the percent distribution of binding. In any event, these observations do not provide a basis for concluding that obesity and increased androgen biological activity are related risk factors for prostate cancer.

The Seventh-Day Adventist Church recommends that its members abstain from meat, consume eggs and cheese sparingly and use milk as a protein source. In the United States, male Seventh-Day Adventists have been found to have a considerably lower prostate cancer mortality rate than the general population (32). Howie and Shultz (33) used 3-d food records to assess the dietary intake of lacto-vegetarian and non-vegetarian Seventh-Day Adventists, and non-Seventh-Day Adventist omnivorous males, all aged 49–62 years. The three groups did not differ in their consumption of fat, but the vegetarians consumed more crude and dietary fiber. Their plasma testosterone and estradiol levels were both significantly lower than those of the other two groups of men and were inversely correlated with the estimates of dietary fiber. We have discussed elsewhere the relationships between dietary fiber and fat, and circulating hormone levels in the context of breast cancer (34).

One study has been published in which men at risk of fatal heart disease were placed on low-fat, low-cholesterol and high-complex carbohydrate diets (35). After 26 d, their serum estradiol concentrations were reduced by an average of approximately 50%; perhaps unexpectedly, there were no changes in the testosterone levels.

ANIMAL MODELS

Attempts to develop animal models for human prostate cancer have met with only limited success and have not really provided a stimulus for experimental studies of dietary factors and prostatic carcinogenesis on a par with those seen in breast cancer research (36–41). Pollard and Luckert (37) observed the spontaneous development of prostate cancers in a genetically susceptible strain of Wistar rats and obtained some indication for a promotional effect of a diet supplemented with linoleic acid (LA)-rich corn oil so as to bring its total fat content to 20% (w/w). When the chemical carcinogen *N*-nitrosomethylurea (NMU) was administered, the prostatic tumor yield was increased, an effect which was enhanced by testosterone administration, and also by a high-fat diet (38,39). However, in another NMU-induced prostate cancer model developed by Bosland *et al.* (40), different levels or types of dietary fat had no promotional effects on tumorigenesis (41).

FATTY ACIDS, GROWTH FACTORS AND PROSTATE CANCER CELL GROWTH

Recent studies in our laboratory have indicated that the fatty acid composition of the diet, as well as the absolute amounts of fat consumed, may influence prostate cancer risk. Our interest in this possibility was stimulated by a combination of epidemiological observations and the results which we had obtained from related investigations into dietary fatty acids and human breast cancer cell growth (42,43).

Prostate and breast cancer incidence rates have both been increasing in the United States, as well as in Japan. We postulated (44), as did others (45,46), that there might be a relationship between the rising breast cancer risk in these countries and an increase in the consumption of vegetable oils rich in LA. Although obviously highly speculative, these arguments may be applied also to carcinoma of the prostate.

As a first approach to the issue, we have performed a series of experiments *in vitro* to examine the effects of both LA, an n-6 polyunsaturated fatty acid (PUFA) and docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), two n-3 fatty acids, on the growth of human prostate cancer cells (47). The PC-3 prostate cancer cell line was responsive to LA, its growth being stimulated in a concentration-dependent manner over a 5–750 ng/mL range when the fatty acid was incorporated into serum-free, bovine serum albumin (BSA) supplemented culture medium. In contrast, the n-3 fatty acids inhibited PC-3 cell growth, an effect which was also dose-dependent and due to an arrest of proliferation rather than nonspecific toxicity. This inhibition *in vitro* was particularly interesting because we had shown previously that the growth of human prostate cancer cells as solid tumors in athymic mice is suppressed by feeding a diet containing (n-3)-rich Menhaden fish oil (48).

There is some epidemiological support for a protective influence of n-3 fatty acids against both prostate and breast cancer. In a study from Japan, where fish traditionally provides the major source of animal protein, Mishina *et al.* (17) reported that a low consumption of seafood was associated with increased prostate cancer risk. Alaskan Eskimo men who eat large quantities of fish are at low risk for prostate cancer, as are Eskimo women for breast cancer (49). To examine this issue further in the context to breast cancer, Kaizer *et al.* (50) made international comparisons and showed an inverse association between percent of calories from fish and disease rates *after* adjustment for dietary fat intake; on the basis of Figures 1 and 2, it is to be expected that this relationship would also apply to prostate cancer.

The n-3 fatty acids compete with LA and its metabolic product arachidomic acid (AA) for enzymes which regulate eicosanoid biosynthesis (Fig. 4). We used pharmacological inhibitors of prostaglandin (PG) and leukotriene (LT) synthesis to demonstrate a requirement for both families of eicosanoids for normal human prostate cancer cell growth *in vitro*, and its stimulation by LA (47). Particularly

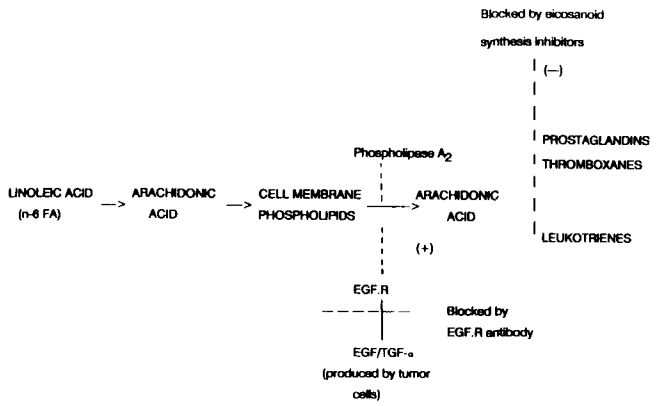

FIG. 4. Interaction of n-6 fatty acids and epidermal growth factor in eicosanoid biosynthesis: sites of metabolic inhibition.

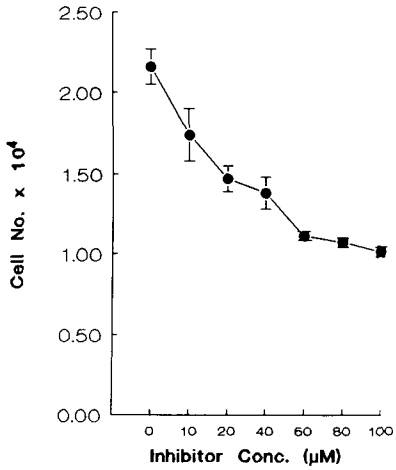

FIG. 5. Suppression of PC-3 human prostate cancer cell growth by caffeic acid, an inhibitor of leukotriene biosynthesis.

significant was the suppressive effect of esculetin (6,7-dihydroxycoumarin) on PC-3 cell growth, because this compound inhibits the lipoxygenase enzymes, and hence LT synthesis, but actually enhances production of the PG (51). As shown in Figure 5, caffeic acid, which has similar effects on LT and PG biosynthesis (52), is also an effective inhibitor of prostate cancer cell growth *in vitro*.

Like PC-3 cells, the DU145 human prostate cancer cell line does not require the presence of androgens for its growth; it grows readily in the absence of serum and synthesizes both epidermal growth factor (EGF) and the related polypeptide transforming growth factor α (TGF-α). These two growth factors, which share the same cell membrane receptors, are secreted into the culture medium by the tumor cells (53). Both PC-3 and DU145 cells possess EGF/TFG-α receptors, and we suggested that an auto-crine loop exists for the regulation of prostate cancer cell growth (47,53). More recently, we obtained direct evidence for such autocrine control by showing that DU145 prostate cancer cell growth is suppressed if the EGF binding sites are rendered inaccessible to the ligands by exposing them to a receptor protein-blocking antibody (54).

After prolonged passage *in vitro*, a subline of DU145 prostate cancer cells was obtained which, like PC-3 cells, is stimulated to grow by LA. Using this subline, we ex-

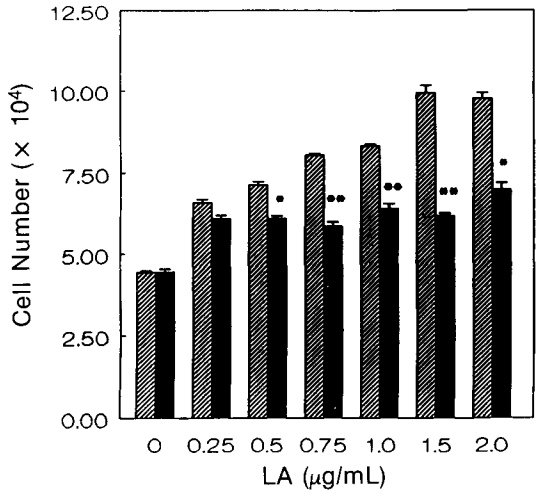

FIG. 6. The stimulation of growth of a DU145 human prostate cancer subline by linoleic acid (cross-hatched blocks), and its inhibition by an antibody (1×10^{-9}M) to the cell membrane epidermel growth factor (EGF) receptors (solid blocks)., The cells were grown in RPMI-1650 plus 1% fetal bovine serum for 3 d. Significant differences between cell number in the presence and absence of the antibody: $^{**}P \oplus 0.001$; $^{*}P \oplus 0.01$).

amined the possibility that a relationship exists between EGF-like polypeptide secretion by the cancer cells and this (n-6)-induced growth response (Fig. 4). When AA is formed from LA, it is largely incorporated into the cell membrane phospholipids where it provides a reserve of substrate for eicosanoid biosynthesis. Mobilization of AA involves phospholipase A_2, an enzyme which has been shown to be stimulated by EGF in BALB/c 3T3 fibroblasts (55,56). Figure 6 shows the concentration-related growth stimulation of the DU145 subline when it was cultured in the presence of LA, and the suppression of this growth response when EGF/TGF-α binding sites were blocked with receptor antibody. Additional experiments will be required to demonstrate that this inhibition is associated with a reduction in eicosanoid biosynthesis and is reversible by high concentrations of exogenous EGF.

COMMENTARY

The epidemiological evidence that dietary fat is involved in determining prostate cancer risk is conflicting, and is of an inferential character. International comparisons show an association between prostate cancer mortality rates and estimates of the consumption of fats of animal, but not vegetable, origin. This distinction holds also for breast cancer (9), but in both cases the association could be a spurious one. More persuasive are the trends in prostate cancer incidence and dietary fat consumption in Japan and the consequences of migration to areas of high prostate (and breast) cancer risk.

There is a need for investigations of dietary composition and prostate cancer risk which are analyzed to distinguish between the effects of fat and energy intakes. This is an important issue and could be examined by appropriately located case-control studies which provide an adequate range of dietary patterns. One such study was performed in northwestern Italy by Toniolo *et al.* (57), and demonstrated a significant association between breast

cancer risk and saturated fat consumption after adjusting for caloric intake.

We have discussed the geographical variation in the prevalence and histological characteristics of prostate cancer pathology at some length because comparative investigations of dietary composition in relation to latent carcinoma of the prostate would be extremely valuable.

Another target for future studies should surely be the large difference in prostate cancer risk for African-Americans and white Americans. A dietary-hormonal hypothesis of prostate cancer etiology is obviously strengthened if it provides a mechanistic explanation for this discrepancy. There is an urgent need for carefully regulated experimental dietary studies into the effects of fat and fiber on male plasma hormone levels. These should include both young men, as a follow-up to the reported differences in serum testosterone levels (28), and middle-aged and older males, many of whom will have preexisting latent carcinomas of the prostate.

Attention should also be focussed on the influence of different types of fatty acids on the biology of human prostate cancer cells. For this work, growth studies *in vitro* should be combined with experiments *in vivo* using athymic nude mice.

It may be considered premature to attempt dietary intervention trials aimed at reducing prostate cancer risk. However, progress is being made in the development of similar trials in breast cancer (58,59), and these will offer the opportunity to include male members of the family in pilot studies and biochemical investigations.

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Dietary Fat and the Development of Pancreatic Cancer[1]

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Pancreatic cancer is the fifth most common cause of death due to cancer. Except for an association with cigarette smoking, its etiology is poorly understood. Because of the dearth of epidemiological clues as to causation, studies with experimental animal models assume greater importance. Rodent models of pancreatic cancer indicate that while dietary fat *per se* does not cause pancreatic cancer, it does enhance or promote tumor development. Subsequent to treatment with a pancreatic carcinogen, high intakes of dietary unsaturated fats of the n-6 series, but not saturated fats, enhance or promote tumor development. A requisite level of linoleic acid is needed for this promotion. Fats of the n-3 series (*e.g.*, certain fish oils) are inhibitory to tumor growth. Promotion by dietary fats appears only partly related to the high caloric content of fat. Mechanistically, certain dietary unsaturated fats appear to selectively enhance the growth rate of carcinogen-induced, pre-cancerous lesions. Irrespective of precise understanding of mechanisms of promotion, it appears possible to intervene in the process of cancer development and reduce the burden of cancer. Experimentally, this may be accomplished by decreasing total fat intake, decreasing caloric intake, increasing exercise or increasing the intake of n-3 fatty acids.
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In the United States, pancreatic cancer ranks eighth with respect to incidence of cancer yet it is the fifth most common cause of death due to cancer with about 24,000 deaths per year (1). Total mortality is approximately equal by sexes and the age-adjusted cancer death rates for both sexes have been essentially unchanged over the last 20 years. Pancreatic cancer is usually diagnosed late in the course of the disease thus precluding effective treatment. The median survival following diagnosis is only a few months and the 5-yr survival is now only 3 to 4% of all cases of pancreatic cancer. The last 20 yr have seen an increase in the 5-yr survival from only 1% to the present levels (1). These survival rates are among the lowest of all forms of cancer. With the exception of an association with the smoking of cigarettes, the etiology of pancreatic cancer is largely unknown (2).

There have been hints that dietary factors are associated with pancreatic cancer. Carroll and Khor (3) identified correlation coefficients between *per capita* consumption of dietary fat in various countries of the world and age-adjusted mortality in those countries from pancreatic cancer to be +0.67 for males and +0.50 for females. While a correlation exists, it is very weak compared to similar observations for breast cancer (+0.94) and colon cancer (+0.93 for males and +0.91 for females). In an independent study using a similar approach, Wynder (4) reached the same conclusion. With correlations possible among many factors including other dietary practices and other nutrients, especially protein intake, this form of epidemiological evidence is not strong. More recent epidemiologic data using the more direct and powerful case-control approach find only a moderate association of pancreatic cancer with the intake of fat or fat-containing foods (5–7).

Because of the dearth of clues as to causation of pancreatic cancer in humans, experimental studies with animal models of this disease assume great importance. For example, results from rodent models indicate that dietary fats are indeed causally related to pancreatic carcinogenesis (8–10) thus adding strength to the relatively weak associations shown from human epidemiological data.

Experimental animal models of human cancers obviously have limitations and such is the case for rodent models of pancreatic carcinogenesis. The two best characterized and most extensively used models of pancreatic cancer are (i) the Wistar or Lewis rat treated with azaserine to produce adenocarcinomas composed predominantly of acinar cells (11) and (ii) the Syrian golden hamster treated with one of several nitrosoamines to yield adenocarcinomas that are predominantly duct-like (12). For two reasons, we have primarily used the rat model of pancreatic cancer. First, the database from other rat models involving both cancer at various sites and nutrition is extremely large and useful. Second, the rat model is quantifiable and suitable to experiments as short as a 4-mon duration.

The rat model is characterized by the early appearance of atypical acinar cell foci and nodules with the subsequent development of adenocarcinomas that predominantly have acinar cell characteristics (11). All evidence indicates that the lesions are of acinar cell origin. Within one month following a single dose of azaserine, foci of atypical acinar cells are commonly observed. Two phenotypically different populations of foci have been identified. Foci of the basophilic phenotype appear to have a low growth potential and are relatively resistant to pancreatic growth stimuli; whereas the acidophilic foci grow throughout the life of the rat and are responsive to various growth stimuli (13). Neither phenotypic population of foci shows signs of regression. Subsequent to a single dose of azaserine (30 mg/kg) to 7-wk-old Lewis rats, carcinomas *in situ* are observed as early as 9 mon, and the incidence of rats with adenocarcinomas increases progressively from 12 to 18 mon (14). Several studies have shown the utility of quantitatively measuring the number and size of the early putative preneoplastic lesions as a predictive index of the ultimate incidence of neoplasms. This quantitative short-term model has been described in detail elsewhere (9,15,16).

Using the rodent model of pancreatic cancer described above, the effects of dietary fats in general, fish oils in particular, and calories and exercise will be discussed.

DIETARY FATS

The first experiments studying dietary fats and pancreatic carcinogenesis in the rat utilized multiple doses of azaserine during an initiation phase of several weeks' duration (17,18). Though multiple doses of an initiating carcinogen are considered less than ideal, multiple

[1]Based on a paper presented at the Symposium on Lipids in Cancer held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

injections of azaserine appear equally as effective as a single dose of the same total quantity of azaserine when individual doses are below the cytotoxic level (19). Weekly doses of azaserine for 5 wks concurrent with feeding diets high in unsaturated fat (20% by weight corn oil) and saturated fat (18% hydrogenated coconut oil and 2% corn oil) resulted in no statistically significant difference between the two high fat diets in either the incidence, multiplicity or severity of pancreatic neoplasms (18). Furthermore, the two groups which were fed high levels of dietary fat did not differ statistically from the control diet group (5% unsaturated fat). Taken together these data indicate that saturated and unsaturated fats fed at a high level have no effect upon the initiation phase of carcinogenesis by azaserine in the rat. Similar conclusions have been drawn for the hamster model of pancreatic cancer (20).

The rat has been extensively used in chronic toxicity and carcinogenicity studies of upwards to two years' duration. Microscopic proliferative lesions of the pancreas, that is, foci of the acidophilic phenotype, have been observed in old rats and the incidence of these lesions were increased significantly when the rats were gavaged with corn oil (21). The quantity of corn oil delivered to these vehicle-control rats in chronic cancer studies increases their fat intake from about 5% to approximately 15% fat by weight in the diet. These foci are observed in pancreases of several rodent species and strains of rats (22) and we have observed increased numbers of these acidophilic foci as rats age (14,19). Though not the only possibility, an attractive explanation of these observations is that dietary unsaturated fat increases the development of "spontaneously" occurring lesions of the pancreas.

The post-initiational enhancement of pancreatic neoplasia by high levels (20% by weight of corn or safflower oil) of dietary unsaturated fats as compared to similar high levels of saturated fats or low (2 to 5%) levels of unsaturated fats in rats was first reported in 1981 (17,18). As discussed above, this phenomenon occurs after chemical carcinogen exposure and is operationally defined as a post-initiational or promotional event (18). Observations from short-term studies with the rat/azaserine model and using the size and number of acidophilic foci as an end point provide many clues regarding the role of dietary fat in pancreatic carcinogenesis. Experiments have shown that from 4% to 8% essential fatty acid (linoleic acid) in diets totaling 20% fat is an approximate threshold for essential fatty acid above which both the number and size of the acidophilic foci grow (23). At essential fatty acid levels below this threshold, the foci do not grow irrespective of the presence of large quantities of dietary fat. Other studies have shown that the [^{3}H]thymidine labeling index, a measure of cell growth, is increased in the acidophilic foci of rats fed unsaturated fat and this increased growth rate is not seen in foci of rats fed either a high dietary level of saturated fat or a low level of dietary unsaturated fat (15). The basophilic foci showed a low labeling index indicative of their low growth potential and presumed general unimportance in the ultimate development of cancers in the rat model.

Again in experiments with the short, 4-mon post-initiation or promotion model, growth of the foci can be modulated upward by feeding a high level (20%) of unsaturated fat to rats which were previously fed a low fat diet and *vice versa* (24). These experiments are similar in concept to the intervention trials by epidemiologists. Basically, halfway through the 4-mon post-initiation phase, the diet is switched from low fat to high fat or *vice versa*. In this latter experiment as well as in previous experiments, these dietary unsaturated fats do not increase the size of the pancreas of non-carcinogen treated controls, thus promotion appears to be relatively specific for the carcinogen-induced foci (25), and spontaneously occurring foci (26).

FISH OILS

A number of studies have indicated that the consumption of n-3 fatty acids may inhibit carcinogenesis. With regard to pancreatic carcinogenesis, only experiments in the rat model have addressed this phenomenon and these experiments have used the 4-mon, short-term foci model. When 20% by weight of the diet was menhaden oil (approximately 7% of the diet being n-3 fatty acids), the number of foci was significantly reduced and no neoplasms were observed (27). Rats fed a similar level of total fat, but of n-6 fatty acids, had a high focal burden and a small but definite incidence of pancreatic neoplasms (27). When all diets were formulated to contain 20% fat, but with various ratios of corn oil to menhaden oil, a threshold in the azaserine-induced focal burden of the pancreas was apparent between 3 and 6% linoleic acid in the diet (28). This is similar to the threshold noted in earlier experiments with this model (23) and in experiments using a model of mammary carcinogenesis (29). While the mechanism by which menhaden oil inhibits pancreatic carcinogenesis is not clear, one observation was that the effect was primarily upon the number of foci observed and not upon the growth of the foci. This implies that the effect occurs early in the post-initiation or promotion phase. Using the intervention concept described above, we have shown that substituting 20% menhaden oil for 20% corn oil in the rat diet for the last 2 mon of the 4-mon experiment significantly suppressed pancreatic foci development. The opposite occurred if rats were switched from menhaden oil to corn oil (28).

CALORIES AND EXERCISE

There is considerable debate over the relative importance of dietary fat intake *per se vs.* the total caloric intake as modulators of carcinogenesis. Numerous investigations have shown that mammary carcinogenesis is enhanced by intakes of high levels of fat especially during the post-initiation phase of carcinogenesis and that there is a minimum level of linoleic acid required for enhancement (29). Since fat is the most calorie dense ingredient in the diet, high fat diets are also calorie-rich. Thus, it has been very difficult to separate the potential effects of fat from calories. Studies by Kirtchevsky *et al.* (30,31) and Boissonneault *et al.* (32) have drawn attention to the importance of calories in the enhancing or promotional effects of dietary fat. It is hypothesized that fat may exert its promotional effects by being more efficiently utilized as an energy source as compared to either protein or carbohydrate. As with many rodent models of cancer, we have shown that restriction of food intake to approximately 90% of the amount consumed by the *ad libitum* fed rats suppressed pancreatic carcinogenesis (17,18).

Recent reports have rekindled an interest in energy utilization as a way of controlling the promotional effects of dietary fats. Cohen *et al.* (33) showed that both caloric restriction and energy utilization (exercise) inhibited the development of *N*-nitrosomethylurea-induced mammary tumors. The exercise group had free access to running wheels and averaged 2.9 km (1.8 miles) per day over the 20-wk experiment. Interestingly, of the rats in the exercise group, the ones which ran the least attained the greatest benefit with respect to protection against mammary tumorigenesis. It is unknown *via* what mechanism voluntary exercise modulates mammary carcinogenesis. Reddy *et al.* (34) observed that for male rats voluntary exercise on running wheels inhibited azoxymethane-induced colon cancer. Similar results in different organ cancer models imply that the mechanism of inhibition of carcinogenesis is not organ specific and must be fairly general in nature. We have observed that voluntary exercise virtually identical to that used by Cohen *et al.* (33) and Reddy *et al.* (34) suppressed pancreatic foci development in our azaserine model (35). The growth of the acidophilic foci are suppressed in the pancreases of the exercise group of rats (35). Thompson *et al.* (36) found that rats initiated with 7,12-dimethylbenz[a]anthracene and exercised on a treadmill actually had enhanced mammary tumor development as compared to the sedentary group. The treadmill activity consisted of running 20 m/min for 15 min/d (0.3 km/d) at a 1% incline for 5 d of the week. Additional studies by Thompson *et al.* (37) indicated the reproducibility of the effect on mammary tumor enhancement and that enhancement occurred with both high (24.6% by weight) and low (5.0%) levels of unsaturated fat, but not when the diet was composed of high levels of primarily saturated fats. Clearly, there are likely to be considerable differences between voluntary and treadmill exercise with regard to the anaerobic and aerobic exercise and the possible stress of exercise. Caution is important, especially when attempting to extrapolate to the human situation.

CONCLUSION

Pancreatic cancer is a serious disease in humans and its causation is not understood. Prevention may be possible even if we do not know the primary cause. Using a rat model of pancreatic cancer, we have shown that diet can retard the development of putative precancerous lesions (acidophilic foci) and adenocarcinomas. A decrease in the total amount of fat, a decrease in calories, an increase in exercise, and a substitution of n-3 fatty acids for n-6 fatty acids will all suppress pancreatic carcinogenesis in the rat. Intervention trials in this rodent model indicate that such dietary alterations can be instituted during the post-initiation phase and need not be "in place" for the entire life of the animal.

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Dietary Fat and Colon Cancer: Animal Model Studies[1]

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Since it was first suggested that high dietary fat is a risk factor in colon cancer, there have been several studies to test this hypothesis. Epidemiologic studies suggested a positive association between dietary fat and colon cancer. Laboratory animal model studies demonstrated that not only the amount of fat, but also types of fat differing in fatty acid composition are important determining factors in colon tumor development. Chemically-induced colon tumor incidence was increased in rats fed the semipurified diets containing 23% corn oil, safflower oil, lard or beef tallow (high-fat) as compared to those fed 5% corn oil, safflower oil, lard or beef tallow diets (low-fat). Diets containing 23% coconut oil, olive oil or fish oil, or high-fat diets containing varying levels of *trans* fat, had no colon tumor-enhancing effect compared to their respective low fat diets. The stage at which the effect of dietary fat is exerted appears to be mostly during the post-initiation phase of colon carcinogenesis. Lack of a colon tumor enhancing effect of dietary fish oil is observed both during the initiation and postinitiation phases. The mechanisms by which various dietary fats increase colon carcinogenesis are not fully understood. In most instances, however, the high-fat diet appears to enhance tumorigenesis through elevation of agents, such as secondary bile acids, that act as promoters of tumor development. Lack of colon tumor promotion by dietary fish oil and *trans* fat appears to be mediated through their effect on mucosal ornithine decarboxylase activity, colonic secondary bile acids and/or prostaglandin synthesis.
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Cancer of the colon is one of the most common cancers in the western countries, including North America, exhibiting more than a tenfold excess when compared to rural populations in Asia, Africa and certain parts of South America. Studies of cancer incidence in high risk areas such as the United States, and low risk countries, such as Japan and Finland, indicate that rates are increasing faster in low than in high incidence areas of the world. It is the second leading cause of cancer deaths in the United States, with estimated 157,500 new cases and about 60,500 deaths in 1991 (1).

Since Wynder *et al.* (2) and Burkitt (3) first suggested that dietary factors in general, and specifically dietary fat and fiber, might play a role in the etiology of colon cancer, a substantial amount of progress has been made in understanding the relationship between nutritional factors and the development of colon cancer in humans. Continuing population studies revealed that the diets particularly high in total fat and low in certain dietary fibers are generally associated with an increased risk of developing colon cancer (4). In addition, dietary fat may be a risk factor in the absence of factors that are protective, such as use of high dietary fiber and fibrous foods (4). Several case-control studies suggest a positive association between dietary fats, especially saturated fat (5,6). A recent prospective study provided evidence that a high intake of animal fat, but not vegetable fat, increases the risk of colon cancer (7). Animal model studies performed in our laboratory and elsewhere have consistently supported human epidemiologic studies and further provided evidence that the colon tumor-promoting effect of dietary fat depends on the type of fat (8). These studies also suggested that the main effect of dietary fat in colon carcinogenesis is during the postinitiation phase of carcinogenesis. Unquestionably, there are areas where more research is necessary and, of course, no single dietary factor and mechanism can account for all colon cancer.

Since several reviews have already appeared on the relationship between dietary fat and colon carcinogenesis, this report will provide a brief overview of current knowledge as to the relationship between the types of dietary fat and colon carcinogenesis in laboratory animal models with emphasis on ω3 fatty acids (fish oil). The basis for the latter point is that epidemiologic studies have found that people who traditionally consume (either regularly or frequently) diets containing fish and marine animals have a lower incidence of several chronic diseases, including cancer, than those who do not or rarely consume such foods (9–11).

AMOUNT OF DIETARY FAT AND COLON CANCER

A variety of compounds, namely 1,2-dimethylhydrazine (DMH), azoxymethane (AOM), methylazoxymethanol (MAM) acetate, 3,2′-dimethyl-4-aminobiphenyl (DMAB) and methylnitrosourea (MNU), that are carcinogenic for the colon have been used in a number of laboratory animal models to investigate the effect of dietary fat on tumorigenesis at this site (12). Although our early experiments (12–14) and those of others (15–18) did not distinguish between the effect of fat on the initiation and postinitiation phases of carcinogenesis, these studies nonetheless indicated a strong relationship between the amount of dietary fat and colon cancer in animal models.

Nigro *et al.* (15) studied the effect of diets containing 5 and 35% beef fat on AOM-induced intestinal tumors in Sprague-Dawley rats. Animals fed the high beef-fat diet developed more intestinal tumors and more metastases in the abdominal cavity, lungs and liver than did the animals fed the low-fat diet. Howarth and Pihl (17) demonstrated that DMH-induced colon tumors were increased in male D/A rats fed a high-fat diet compared with those fed a low-fat diet. Studies conducted in our laboratory indicate that animals fed 20% lard or corn oil diets were more susceptible to DMH-induced colon tumors compared with those fed 5% lard or corn oil diets. The type of fat

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Abbreviations: AA, arachidonic acid; AOM, azoxymethane; DHA, docosahexaenoic acid; DMAB, 3,2′-dimethyl-4-aminobiphenyl; DMH, 1,2-dimethylhydrazine; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MAF, methylformamide; MAM, methylazoxymethanol; MNU, methylnitrosourea; ODC, ornithine decarboxylase; PG, prostaglandins.

appears to be immaterial at the 20% level; however, at the 5% fat level, unsaturated fat (corn oil) induced more colon tumors than did saturated fat (lard) (13). Pence and Buddingh (18) reported that DMH-induced colon tumors were increased in male F344 rats fed a high-fat diet as compared with those fed a low-fat diet. A recent study by Sakaguchi *et al.* (19) demonstrated a significantly higher incidence of colon tumors in rats fed 5% linoleic acid diet than in those fed 4.7% stearic acid plus 0.3 linoleic acid diet.

Investigations also were carried out to test the effect of diets comprising 20% and 5% beef fat on colon carcinogenesis by a variety of carcinogens which differ in metabolic activation, namely DMH, MAM acetate, DMAB, or MNU (14,20). In these studies, semipurified diets containing high and low levels of fat were fed to animals before, during and after carcinogen treatment to study the specific effects on the initiation and promotion stages of colon carcinogens. Combined results of these two studies (14,20) indicate that, irrespective of the nature of the carcinogens, animals fed the diet containing a high amount of beef fat had a greater incidence of colon tumors than did rats fed a low beef-fat diet.

There are studies, however, which did not show an enhancing effect of dietary fat in colon carcinogenesis (21,22). Results from the laboratory of Nauss (21,22) indicated that 20% beef fat or corn oil had no colon tumor enhancing effect. Animals fed a diet which promoted optimal growth had a significant increase in colon tumor incidence, but not in frequency or size. This effect was not observed using a second diet, which resulted in slower growth.

TYPE AND AMOUNT OF DIETARY FAT DURING INITIATION AND POSTINITIATION PHASES

The pioneering study of Bull *et al.* (23) indicated that ingestion of a high amount of beef fat increased the intestinal tumor incidence in rats when fed after AOM treatment, but not during or before the carcinogen administration, suggesting that excess dietary beef fat acts during the postinitiation phase of colon carcinogenesis. We investigated the effect of various levels of polyunsaturated (corn oil) and saturated (lard) fats fed during the initiation or postinitiation phases of colon carcinogenesis (24). When the animals were fed the diets containing 23.5% corn oil during the stage of initiation, there was no increase in the incidence of colon tumors as compared with that of animals on a 5% corn oil diet. When the 23.5% corn oil diet was fed during the postinitiation stage of carcinogenesis, there was a significant increase in colon tumor incidence compared with that of animals fed the 5% corn oil diet. These results suggest that the effect of a high corn oil diet in colon carcinogenesis is observed mainly during the postinitiation stage, rather than during the initiation stage of carcinogenesis. On the other hand, animals fed the 23.5% lard diet during the initiation or postinitiation phase had a higher colon tumor incidence as compared to those fed 5% lard diet, suggesting the importance of type of dietary fat during the initiation and postinitiation stages of colon carcinogenesis.

The effect of high-fat diets containing coconut, olive, safflower and corn oil fed during the postinitiation phase of colon carcinogenesis also was investigated in rats (25). As expected, the animals fed the high corn oil and safflower oil (23.5%) diets had a higher incidence of AOM-induced colon carcinogenesis than did those fed the diets low in their respective fats (5%). In contrast, high-fat diets containing coconut oil or olive oil had no colon tumor enhancing effect.

In another study, we investigated the effect of various levels of *trans* fat on AOM-induced colon carcinogenesis in female F344 rats (26). The experimental high-fat diets containing 23.5% corn oil, 5.88% *trans* fat + 5.88% corn oil + 11.76 oleinate (low *trans* fat), 11.76% *trans* fat + 5.88% oleinate (intermediate *trans* fat), and 5.88% corn oil + 17.64% *trans* fat (high *trans* fat) were fed during the postinitiation phase of colon carcinogenesis. The results indicate that the high-fat diets with increasing levels of *trans* fat had no significant effect on colon tumor incidence. Animals fed the high-fat diets containing various levels of *trans* fat have a lower colon tumor incidence as well as multiplicity of adenocarcinomas compared to those fed the diet containing high corn oil (23.5).

The lack of colon tumor promoting effect of high dietary olive oil, coconut oil and *trans* fat in contrast to that of high dietary corn oil, safflower oil, beef fat and lard suggests that the fatty acid composition of a dietary fat is one of the determining factors in colon carcinogenesis. The stage of carcinogenesis at which the effect of dietary fat is exerted appears to be mostly during the promotional phase of carcinogenesis, rather than during the initiation phase.

ω3 FATTY ACIDS (FISH OIL) AND COLON CANCER

The high levels of highly polyunsaturated ω3 fatty acids such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) present in marine oils make them unique dietary fats. Most common animal and vegetable fats are virtually devoid of these fatty acids, except for perilla oil which contains high levels of α-linoleic acid, an ω3 fatty acid (18:3n-3). Most commonly consumed vegetable oils in the United States, such as corn oil and safflower oil, contain high levels of polyunsaturated fatty acids of ω6 type, *e.g.*, linoleic acid (18:2n-6) (Table 1).

Interest in the potentially beneficial effect of marine lipids originated by a reportedly lower incidence of thrombotic and immunologically-mediated diseases in Greenland Eskimos when compared with mainland Danish population (27). A low prevalence of cardiovascular disease has been observed in Eskimos, who eat a mostly fish diet, a high source of ω3 fatty acids (27,28). The Greenland Eskimo diet was found to contain more protein and less carbohydrates than average Danish foods and an almost equal amount of fat (27). It is also well known that Japanese people have a dietary habit of eating fish as a main source of protein and that incidence of thrombotic cardiovascular disease in Japan is lower than that in western countries. A recent epidemiologic study by Hirai *et al.* (10) on the intake of fish and the mortality rate of ischemic heart disease and cardiovascular disease indicates a lower mortality rate in the fishing area of Japan, where the intake of fish was about 260 g/day, than in the farming area of Japan, where the fish intake was about 90 g/day.

Interest in marine oils also emerged from the findings that cancer incidence rates are generally low in Eskimos

TABLE 1

Fatty Acid Composition of Dietary Fats (%)

Fatty acids	Corn oil	Safflower oil	Soybean oil	Peanut oil	Butter	Lard	Perilla oil	Menhaden oil
4:0	—	—	—	—	3.2	—	—	—
6:0	—	—	—	—	1.9	—	—	—
8:0	—	—	—	—	1.1	—	—	—
10:0	—	—	—	—	2.5	0.1	—	—
12:0	—	—	—	—	2.8	0.5	—	—
14:0	—	0.2	0.1	0.1	10.1	1.4	0.1	8.4
16:0	11.5	9.5	12.3	9.5	26.3	23.7	8.3	15.2
18:0	2.2	2.6	3.9	2.3	12.1	13.0	1.9	2.7
16:1	—	0.3	0.4	—	2.3	2.6	0.4	11.6
18:1n-9	22.6	12.3	21.6	45.6	25.1	40.9	14.3	9.5
18:2n-6	58.7	74.0	52.6	31.0	2.3	10.0	15.9	1.8
18:3n-3	0.8	0.9	8.2	—	1.4	1.4	58.2	1.8
20:5n-3	—	0.1	—	—	—	—	0.3	16.0
22:5n-3	—	trace	0.2	—	—	—	0.1	3.9
22:6n-3	—	—	0.1	—	—	—	0.2	10.8

of Alaska and Greenland as compared to North Americans and other western population groups who consume diets containing high amounts of fat (9,29–31). In Greenland and Iceland, a rise in the incidence of breast cancer followed a period when the dietary habits became westernized, changing from a typical fish diet to one containing decreased amounts of ω3 fatty acids (29). A recent report on prostatic cancer in Japan indicated that, among other factors, the risk for the development of prostate cancer was higher in individuals whose diets contained little or no seafood (32). These studies resulted in an interest in the role of fish oil in health and disease.

Studies conducted in our laboratory on the relationship of dietary fish oil and colon cancer revealed that fish oil protects against chemically-induced colon carcinogenesis (33,34). In the first study, we tested the effect of diets containing 5% corn oil, 23.5% corn oil, 3% menhaden oil + 1% corn oil or 22.5% menhaden oil + 1% corn oil fed during postinitiation phase on AOM-induced colon carcinogenesis in male F344 rats (33). The results indicated that the colon tumor incidence (% animals with tumors) and multiplicity (number of tumors/animal) were significantly lower in animals fed low and high menhaden oil diets than in animals fed the high corn oil diet, suggesting that high intake of dietary fish oil had no colon tumor promoting effect (Table 2).

Although high dietary fish oil inhibited colon carcinogenesis in the above study (33), large amounts of fish oil may induce a variety of pathophysiological conditions and harmful side effects. Therefore, another study was conducted to investigate the efficacy of varying amounts of menhaden oil and corn oil on colon carcinogenesis to determine the optimum dietary levels at which the combination of two sources of fat elicits maximum inhibition when fed during the postinitiation phase of carcinogenesis (34). In this study, in addition to 5% corn oil (5% CO) and 1% corn oil + 4% menhaden oil (1% CO + 4% MO) diets, high-fat diets containing 23.5% corn oil (23.5% CO), 17.6% corn oil + 5.9% menhaden oil (17.6% CO + 5.9% MO), 11.8% corn oil + 11.8% menhaden oil (11.8% CO + 11.8% MO) or 5.9% corn oil + 17.6% menhaden oil (5.9% CO + 17.6% MO) were tested (Table 3). Feeding of high-fat diets containing 17.6% CO + 5.9% MO, 11.8% CO + 11.8% MO or 5.9% CO + 17.6% MO significantly inhibited the incidence of colon tumors as compared to 23.5% CO.

The fatty acid composition of the microsomal fraction of colonic mucosa and tumors was also measured in the animals fed the experimental diets. This study demonstrated that feeding increasing levels of menhaden oil altered the incorporation of fatty acids into the microsomes of colonic mucosal cells and tumors (Tables 4 and 5). The monounsaturated and saturated fatty acids in colonic mucosa were unaffected by feeding various levels of corn oil and menhaden oil, whereas the polyunsaturated

TABLE 2

Azoxymethane-Induced Colon Carcinogenesis in Male F344 Rats Fed ω3 Fatty Acid-Rich Diets During Postinitiation Phase

Experimental diets	% Animals with colon tumors
5% CO[a]	54[b]
23.5% CO	92
4% MO + 1% CO	50[b]
22.5% MO + 1% CO	33[b]

[a]CO, corn oil; MO, menhaden oil.

[b]Significantly different from the 23.5% CO diet, $P < 0.05$.

TABLE 3

ω6 and ω3 Fatty Acid Composition of Experimental Diets and AOM-Induced Colon Tumors

Experimental diets[a]	Fatty acids (%)		Colon tumor incidence (% animals with tumors)
	ω6	ω3	
5% CO diet	2.8	0	70[b]
1% CO + 4% MO diet	0.6	1.1	59[b]
5.9% CO + 17.6% MO diet	3.6	4.8	63[b]
11.8% CO + 11.8% MO diet	6.7	3.2	63[b]
17.6% CO + 5.9% MO diet	10.0	1.6	70[b]
23.5% CO diet	13.1	0	93

[a]Modified AIN-76 diet; CO, corn oil; MO, menhaden oil.

[b]Significantly different from the 23.5% CO diet, $P < 0.05$.

TABLE 4

Percentage Composition of Fatty Acids in the Microsomal Fraction of Colonic Mucosa of Male F344 Rats Fed the Experimental Diets[a]

	Corn oil diets		Menhaden oil diets			
Fatty acids	5% CO	23.5 CO	1% CO + 4% MO	17.6% CO + 5.9% MO	11.8% CO + 11.8% MO	5.9% CO + 17.6% MO
Palmitic acid	14.1 ± 1.6^{b}	13.9 ± 2.0^{b}	13.6 ± 2.1^{b}	14.4 ± 2.0^{b}	14.9 ± 2.3^{b}	15.4 ± 2.5^{b}
Palmitoleic acid	1.5 ± 0.1^{b}	1.8 ± 0.3^{b}	3.9 ± 1.0^{b,c}	2.4 ± 0.4^{b}	3.6 ± 0.5^{c}	4.0 ± 0.3^{c}
Stearic acid	20.6 ± 3.4^{b}	21.9 ± 3.0^{b}	21.4 ± 3.0	19.6 ± 4.1^{b}	19.2 ± 4.0^{b}	19.6 ± 3.4^{b}
Oleic acid	12.6 ± 2.4^{b}	11.4 ± 1.9^{b}	15.4 ± 3.0^{b}	15.9 ± 2.2^{b}	14.4 ± 3.1^{b}	15.1 ± 3.4^{b}
Linoleic acid	26.4 ± 3.4^{b}	26.6 ± 2.8^{b}	10.1 ± 2.0^{d}	20.4 ± 2.4^{e}	18.2 ± 1.8^{e}	15.2 ± 2.8^{e}
Linolenic acid	1.9 ± 0.2^{b}	2.2 ± 0.3^{b}	0.4 ± 0.1^{c}	1.6 ± 0.2^{c}	1.0 ± 0.2^{c}	0.6 ± 0.1^{c}
Arachidonic acid	16.5 ± 2.4^{b}	19.6 ± 2.0^{b}	3.1 ± 0.2^{c}	6.4 ± 0.5^{d}	5.8 ± 0.6^{d}	5.7 ± 0.7^{d}
Eicosapentaenoic acid	0.2 ± 0.1^{b}	0.3 ± 0.2^{b}	16.8 ± 2.0^{c}	10.5 ± 1.1^{d}	11.5 ± 1.4^{d}	11.9 ± 2.0^{d}
Docosapentaenoic acid	0.2 ± 0.1^{b}	0.2 ± 0.1^{b}	2.0 ± 0.2^{c}	1.8 ± 0.2^{c}	1.5 ± 0.2^{c}	1.8 ± 0.3^{c}
Docosahexaenoic acid	0.3 ± 0.1^{b}	0.4 ± 0.2^{b}	8.9 ± 0.9^{c}	5.8 ± 09.7^{d}	7.4 ± 0.7^{c}	7.1 ± 0.6^{c}
Others	5.5	1.7	4.4	1.2	2.5	3.6

[a]CO, corn oil; MO, menhaden oil. Mean ± SE (n = 6). Superscripts b–e: Means in the same horizontal column that do not share a common superscript are significantly different at $P < 0.05$ (analysis of variance).

TABLE 5

Percentage Composition of Fatty Acids in the Microsomal Fraction of Colonic Tumors of Male F344 Rats Fed the Experimental Diets[a]

	Corn oil diets		Menhaden oil diets			
Fatty acids	5% CO	23.5 CO	1% CO + 4% MO	17.6% CO + 5.9% MO	11.8% CO + 11.8% MO	5.9% CO + 17.6% MO
Palmitic acid	15.4 ± 1.6^{b,c}	14.6 ± 1.4^{b}	16.8 ± 2.0^{c}	18.7 ± 2.1^{c}	17.4 ± 1.8^{c}	18.1 ± 2.0^{c}
Palmitoleic acid	2.1 ± 0.2^{b}	1.9 ± 0.2^{b}	5.2 ± 0.20^{c}	2.8 ± 0.4^{b}	5.0 ± 0.6^{c}	6.4 ± 0.8^{c}
Stearic acid	13.8 ± 1.1^{b}	12.9 ± 1.0^{b}	13.6 ± 1.4^{b}	14.6 ± 1.8^{b}	14.0 ± 1.2^{b}	12.9 ± 1.4
Oleic acid	14.1 ± 1.2^{b}	14.2 ± 1.5^{b}	16.9 ± 1.5^{b}	16.4 ± 1.6^{b}	19.8 ± 1.9^{c}	19.7 ± 1.6^{c}
Linoleic acid	10.0 ± 1.1^{b}	13.6 ± 1.2^{c}	5.4 ± 0.8^{d}	12.6 ± 1.1^{c}	4.1 ± 0.3^{e}	3.0 ± 0.2^{g}
Linolenic acid	0.61 ± 0.1^{b}	0.69 ± 0.1^{b}	0.62 ± 0.2^{b}	0.74 ± 0.2^{b}	0.54 ± 0.2^{b}	0.60 ± 0.3^{b}
Arachidonic acid	11.0 ± 1.2^{b,c}	12.4 ± 1.1^{b}	9.6 ± 0.9^{b}	10.5 ± 1.3^{b,c}	6.4 ± 0.5^{d}	4.2 ± 0.5^{e}
Eicosapentaenoic acid	0.2 ± 0.1^{b}	0.2 ± 0.1^{b}	4.8 ± 0.1^{c}	1.7 ± 0.1^{d}	3.5 ± 0.3^{e}	5.1 ± 0.5^{c}
Docosapentaenoic acid	1.0 ± 0.1^{b}	0.8 ± 0.1^{b}	1.6 ± 0.2^{c}	2.0 ± 0.3^{c}	3.0 ± 0.4^{d}	3.2 ± 0.5^{d}
Docosahexaenoic acid	1.9 ± 0.2^{b}	2.2 ± 0.3^{b,c}	5.8 ± 0.3^{d}	3.0 ± 0.4^{d}	6.4 ± 0.5^{d}	7.2 ± 0.5^{d}
Others	29.1	26.5	19.7	17.0	19.9	19.6

[a]Mean ± SE (n = 6). CO, corn oil; MO, menhaden oil. Superscripts b–g: Means in the same horizontal column that do not share a common superscript are significantly different at $P < 0.05$.

fatty acids were modified by dietary corn oil and menhaden oil (Table 4). Compared to microsomes prepared from rats fed the 23.5% CO diets, microsomes from the animals fed the menhaden oil diets were enriched with ω3 fatty acids. The increasing levels of menhaden oil in the diet significantly increased the ω3 fatty acids, namely EPA, docosapentaenoic acid (DPA) and DHA, and decreased the ω6 fatty acids such as linoleic and arachidonic acid (AA). Significant differences also were observed in the polyunsaturated fatty acid composition of microsomal lipids of colonic tumors among the animals fed the various levels of corn oil and menhaden oil (Table 5). Increasing levels of menhaden oil in the high-fat diets decreased the linoleic acid, linolenic and AA, but increased EPA, DPA and DHA. There was also a significant increase in monounsaturated fatty acids, namely palmitoleic acid and oleic acid, with increasing levels of menhaden oil in the high-fat diets.

The phenomenon of initiation-promotion as stages of carcinogenesis developed for skin has been observed in a variety of different organs. Since many carcinogens require metabolic activation in liver and other organs, including colon, to exert their carcinogenic effects, and enzyme systems that metabolize carcinogens have been shown to be influenced by dietary fats, it is likely that different types of fat may have an effect during the initiation stage in colon cancer. Studies conducted in our laboratory demonstrated that F344 rats fed the high-fat diet containing lard during the initiation or postinitiation phase enhanced the development of colon adenocarcinomas, whereas feeding of the high corn oil diet had an enhancing effect only during the postinitiation stage of colon carcinogenesis (24). These results suggest that the effect of dietary fat during the initiation phase of colon carcinogenesis depends on the types of fat and their fatty acid composition.

We have designed a study to investigate the modulating effect of high fish oil diet rich in ω3 fatty acids when fed during the initiation phase of colon carcinogenesis (35). In this study, five-week-old male F344 rats were divided into seven groups (39 rats/group) and fed the semipurified diets containing 5% corn oil (LCO), 23.5% corn oil (HCO) or 18.5% menhaden oil plus 5% corn oil (HFO). At seven weeks of age, all animals except the vehicle (normal saline)-treated groups were given two weekly *s.c.* injections of AOM at a dose rate of 15 mg/kg body weight, once weekly. Three days after the second injection of AOM, groups of animals fed LCO, LCO, HCO, HCO, HCO, HFO or HFO diets were transferred to LCO, HCO, LCO, HCO, HFO, HCO or HFO, respectively, and continued on these diets until termination of the experiment (Table 6). All animals were necropsied 42 weeks after carcinogen treatment. As expected, the HCO diet fed during the postinitiation period significantly increased the AOM-induced incidence and multiplicity of colon tumors, whereas the HCO diet fed during the initiation phase of carcinogenesis had no effect (Table 6). Colon tumor incidence and multiplicity were significantly reduced in groups fed the HFO diet either at initiation and/or postinitiation phases of carcinogenesis as compared to those fed the HCO diet (Table 6).

A recent study demonstrated a protective effect of menhaden oil on DMH-induced colon carcinogenesis in mice (36). In addition to menhaden oil, the modulating effect of EPA and perilla oil rich in α-linolenic acid (18:3n-3)

TABLE 6

Azoxymethane-Induced Colon Carcinogenesis in Male F344 Rats Fed ω3 and ω6 Fatty Acids During Initiation and Postinitiation Phases

Group	Experimental diets fed[a]		% Animals with colon tumors
	Initiation	Postinitiation	
1	LCO	LCO	47[b]
2	LCO	HCO	97
3	HCO	LCO	53[b]
4	HCO	HCO	97
5	HCO	HFO	50[b]
6	HFO	HCO	50[b]
7	HFO	HFO	23[b,c]

[a]LCO, low corn oil; HCO, high corn oil; HFO, high fish oil.
[b]Significantly different from groups 2 and 4, $P < 0.05$.
[c]Significantly different from groups 3, 5 and 6, $P < 0.05$.

in colon carcinogenesis has been studied in laboratory animal models. Minoura *et al.* (37) compared the AOM-induced colon tumor promoting or inhibitory effect of dietary EPA and linoleic acid in male Donryu rats. Animals fed the EPA diet (4.7% EPA + 0.3% linoleic acid) had a significantly lower colon tumor incidence as compared to those fed the 5% linoleic acid diet. In addition, the EPA diet suppressed the excessive production of prostaglandin (PG) E_2, whereas linoleic acid diet caused a marked increase in the tumor content of PGE_2. In another study, Hirosa *et al.* (38) demonstrated that the dietary perilla oil significantly inhibited AOM-induced colon carcinogenesis and DMBA-induced mammary carcinogenesis in female Sprague-Dawley rats. In the other organs examined, the incidence of kidney nephroblastomas was lower in animals fed the perilla oil diet.

POSSIBLE MECHANISMS OF DIETARY FAT IN COLON CANCER

Various mechanisms have been proposed to explain the promoting effect of a high corn oil diet. These mechanisms include high-fat-induced alterations of tumor-promoting secondary bile acid (deoxycholic acid and lithocholic acid) content of the gut and of cell proliferation as indicated by an increase in colonic mucosal ornithine decarboxylase (ODC) activity (9). Dietary fat increases the concentration of colonic secondary bile acids, as well as modifies the activity of gut microflora which, in turn, enhances the formation of tumor promoting compounds in the colon (12). These secondary bile acids have been shown to act as tumor promoters in the colon (12). Takano *et al.* (39) demonstrated that the induction of colonic epithelial ODC activity by bile acids may play a role in these mechanisms. Increased levels of ODC and of polyamines have been found in tissues undergoing rapid growth and differentiation and in tumor promotion. Our studies (12) and those of Rozhin *et al.* (40) indicate that high dietary corn oil or beef fat increases colonic ODC levels. In addition, there is evidence that activation of protein kinase C by bile acids in colonic epithelium may represent a critical intracellular event in the process by which bile acids provoke a proliferative response (41). Bile acids induce release of arachidonate from colon and the conversion of arachidonic acid to PG (41). With regard to the lack of tumor-promoting

effect of high dietary fish oil fed during the postinitiation phase in comparison to high dietary corn oil (Table 6), several mechanisms, including eicosanoid metabolism, have been proposed. In our study, high corn oil and fish oil diets and a low corn oil diet contained, respectively, about 13.2, 3.5 and 2.8% linoleic acid, a precursor for PG synthesis. EPA and DHA present in fish oil have been shown to inhibit oxidative metabolism of AA by the cyclooxygenase pathway that is involved in PG synthesis (42,43). EPA is also a competitive inhibitor of the lipoxygenase pathway. Recent studies have demonstrated that certain PG synthesis inhibitors such as indomethacin and piroxicam inhibit colon carcinogenesis (44-46). It is therefore possible that the lack of colon tumor promoting effect of a high fish oil diet in comparison to a high corn oil diet might be due to alterations in PG synthesis.

The difference in colon carcinogenesis between the high dietary fish oil as compared to high corn oil when fed during the initiation phase (Table 6) may be related to the action of $\omega 3$ fatty acids on the metabolic activation and detoxification of AOM. The metabolic activation of AOM to a reactive species capable of alkylating DNA occurs in two steps: (i) the hydroxylation of AOM to MAM; and (ii) the oxidation of MAM to the highly unstable methylazoxyformamide (MAF) (47,48). Whereas hydroxylation of AOM to MAM was found to occur in the rat liver (47), the oxidation of MAM to MAF is affected by microsomes from rat liver and colon (48), as well as by alcohol dehydrogenase from the cytosol of both these organs (49). It is likely that $\omega 3$ fatty acids might increase the rate of detoxification of AOM, which could result in decreased delivery of MAM to the colon *via* the bloodstream, thereby reducing AOM-induced colon carcinogenesis during the initiation phase.

In summary, the results thus far generated in laboratory animal models indicate that the colon tumor promoting effect of high dietary fat depends on the type of fat and its fatty acid composition. High-fat diets containing corn oil, safflower oil, beef tallow and lard have been shown to enhance colon tumorigenesis, whereas the high-fat diets containing olive oil, *trans* fat and fish oil have no such enhancing effect. Potential mechanisms for these varied effects have not been completely identified. Future research is indicated for the identification of the mechanism of dietary fat-mediated colon carcinogenesis at the molecular level.

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Humans, Lipids and Evolution[1]

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The genetically ordered physiology of contemporary humans was selected over eons of evolutionary experience for a nutritional pattern affording much less fat, particularly less saturated fat. Current dietary recommendations do not accord exactly with those generated by an understanding of prior hominoid/hominid evolution. Similarly, widely advocated standards for serum cholesterol values fail to match those observed in recently studied hunter-gatherers, whose experience represents the closest living approximation of "natural" human lipid metabolism. The evolutionary paradigm suggests that fats should comprise 20-25% of total energy intake, that the ratio of polyunsaturated to saturated fat should exceed 1.0, and that total serum cholesterol levels should be below 150 mg/dL (~4 mM/L).
Lipids 27, 814-820 (1992).

The current human gene pool appears to be of ancient origin. Mammalian oncogenes are functionally homologous to those of yeast and house flies (1,2); humans and chimpanzees are only 1.6% different, genetically, even though our ancestral lines diverged at least five million years ago (3); and dental analysis suggests that Africans, Asians and Europeans are more like their ancestors of 15,000 years ago than they are like each other (4). Such considerations make it likely that the collective human genome has changed only minimally since anatomically modern *H. sapiens* became widespread, approximately 40,000 years ago.

One of the most important influences affecting genetic selection and adaptation is the interaction between a species and its food supply. For nearly all our line's existence, evolutionary forces maintained an appropriate relationship between our ancestors' metabolism and their diet. However, since the Agricultural Revolution of 10,000 years ago and, particularly, following the Industrial Revolution of the late eighteenth century, genetic evolution has been quite unable to keep pace with changes in human living conditions, including our altered nutritional patterns.

The diets of late Stone Age humans must have varied greatly with latitude and season; certainly there was no one "paleolithic diet" just as there is no one "American diet." However, as the average dietary intake of people in industrialized Western nations can be described and statistically characterized, so can that of our preagricultural ancestors. Their earlier experience has continuing relevance because the Late Paleolithic, from about 35,000 to 15,000 years ago, may be considered the latest time period during which the collective human gene pool functioned within the nutritional parameters for which it had been originally selected. It is quite likely that our physiology today is designed for a Stone Age diet.

Diets in current affluent nations deviate in many important respects from those of preagricultural societies: differences in our intake of sodium, fiber, protein, calcium, refined carbohydrate, vitamins and alcohol variously affect our growth, reproduction and health. Changes in dietary fat patterns, with their resultant impact on lipid metabolism and endocrine function, are perhaps most important of all.

HUMAN FOOD SOURCES

Animal Foods

Wild game. The meat which formed a major part of human diets for the past million years was predominantly wild game. Neither fowl nor fish became common dietary constituents until about 20,000 years ago (5), so the main nutritional adaptation over this period was to red meat.

It was during the transition from *Homo habilis* to *H. erectus,* an evolutionary stage which occurred in Africa approximately 1.7 million years ago, that meat consumption assumed increased importance. *H. erectus* remained largely confined to the African continent until about one million years ago when migration into the tropical, subtropical and even the warmer temperate regions of Asia and southern Europe began. The geographical origin of anatomically modern humans, *H. sapiens,* is currently debated, but both archeological remains and studies of mitochondrial DNA are beginning to indicate that the earliest truly modern humans evolved between 200,000 and 100,000 years ago, probably in Africa.

Accordingly, the ancestors of today's humans consumed African game animals as a substantial part of their diet for over 1.5 million years. The nutritional composition of free-living African mammals is, on average, reasonably similar to that of wild game from other continents (Table 1) but, because animals in tropical regions seldom hibernate and have less need for thermal insulation, there is less seasonal variation in their fat content than in animals in the colder portions of temperate zones (7).

Commercial meat. For over a century, one of the major goals of "scientific" animal husbandry was to increase the carcass fat content of animals raised for consumption. The economic importance of carcass fat content is evident in the official grading system—those animals with the highest proportion of fat to lean are labeled "prime" and command the highest prices; those with the least fat are graded "utility" and are not used for human consumption. In the past few years meat producers have begun to reverse this trend, but the most recent U.S. Department of Agriculture figures reveal the extent to which the past century's efforts succeeded and how nutritionally different the animals we consume are from those available to our pre-industrial ancestors (Table 2).

Although some of the fat in commercial meat carcasses is intramuscular (the marbling which makes meat tender), most is "separable," lying between muscles and as

[1] Based on a paper presented at the Symposium on Lipids in Cancer held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

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Abbreviation: P/S, polyunsaturated/saturated fatty acid ratio.

TABLE 1

Nutritional Properties of Wild Game

Continent and examples	Energy (kcal/100 g)	Protein (g/100 g)	Fat (g/100 g)	Cholesterol (mg/100 g)	Polyunsaturated (as % all fatty acids)	ω3 Fatty acids (as % all fatty acids)	References
Africa							
Topi	106.0	20.2	2.1		35		6-10
Cape buffalo	140.0	19.3	6.3		34	1.9	6,8,9
Wildebeest	129.0	19.4	4.9		30		6,7,9,11
Waterbuck	93.0	20.3	1.8		35		6,7,9
Eland	126.0	19.6	4.8		36		6,8-10
Mean ± SE	132.2 ± 4.1	19.9 ± 0.1	3.7 ± 0.5		35.6 ± 1.6	1.9	
(n)	(15)	(15)	(15)		(7)	(1)	
North America							
Elk	111.5	22.7	1.8	61.3	25.3	3.6	12-15
Moose	125.0	27.1	1.6	59.0	39.3		7,15-17
Seal	145.0	28.6	4.0			6.2	16-19
Bison	105.0	26.4	2.8	48.1	14.7	2.5	7,12,14,15,20,21
Deer	142.7	23.7	4.7	81.0	22.5		7,14,15,17,22
Horse	127.7	20.8	3.7	62.0	30.9		15,23-25
Mean ± SE	144.5 ± 7.8	24.1 ± 0.7	4.8 ± 0.9	69.5 ± 5.0	23.6 ± 2.4	3.8 ± 0.7	
(n)	(19)	(19)	(19)	(11)	(13)	(6)	
Australia							
Water buffalo	126.5	20.7	2.9	46.0	28.3	1.8	15,23,26
Goanna lizard	183.0	34.3	4.6				26
Kangaroo			1.3	54.0	40.0	1.7	23,24,27,28
Mean ± SE	149.4 ± 21.2	27.0 ± 3.2	3.4 ± 1.2	52.7 ± 3.5	33.1 ± 3.5	1.9 ± 0.2	
(n)	(4)	(4)	(5)	(3)	(3)	(3)	
Total series							
Mean ± SE	132.7 ± 5.3	22.7 ± 0.6	4.2 ± 0.5	65.9 ± 4.4	28.5 ± 1.9	3.1 ± 0.5	
(n)	(38)	(38)	(39)	(14)	(23)	(10)	

TABLE 2

Nutritional Properties of Commercial Meat

	Energy (kcal/100 g)	Protein (g/100 g)	Fat (g/100 g)	Cholesterol (mg/100 g)	Polyunsaturated (as % all fatty acids)	References
Lean + fat						
Lamb	267	16.9	21.6	72	8.5	15
Veal	144	19.4	6.8	82	8.2	15
Pork	275	16.7	22.6	72	11.3	29
Choice beef	289	17.5	23.8	70	4.1	30
Regular ground beef	310	16.6	26.6	85	4.6	30
Regular ham	182	17.6	10.6	57	12.7	29
Mean ± SE	244 ± 26.9	17.5 ± 0.4	18.7 ± 3.3	73.0 ± 4.1	8.2 ± 1.4	
Lean only						
Lamb	134	20.3	5.3	65	10.7	15
Veal	112	20.2	2.9	83	14.4	15
Pork	147	20.2	6.8	65	11.6	29
Choice beef	149	20.9	6.6	60	4.7	30
Extra lean ground beef	234	18.7	17.1	69	4.8	30
Extra lean ham	131	19.4	5.0	47	10.8	29
Mean ± SE	151.2 ± 17.4	20.0 ± 0.3	7.3 ± 2.0	64.8 ± 4.8	9.5 ± 1.6	

independent deposits elsewhere in the carcass. Neither component is as prominent in African game. Ledger (6) agrees, writing "In (African) game animals . . . high yields of lean . . . result from higher lean constants (carcass muscular tissue as a percentage of live weight) . . . there was no evidence these animals compensated the low carcass fat by increased deposits of internal fat (pericardial, omental, and mesenteric). The low intramuscular fat (marbling) in game animals was consistent with their low total fat content . . . the roundness of form so often

referred to in the description of condition of tropical game animals as 'fat' is a misnomer." The actual amount of fat ingested by individuals eating meat from Western supermarkets depends to an important degree on how much or how little of the separable fat is cleaned from the muscle tissue before consumption. This was a factor of relatively little significance for our African ancestors and, to a lesser extent, for all people prior to the twentieth century.

Recent breeding and feeding practices presumably account for the reduced proportion of polyunsaturated fat in commercial meat. The structural fats of mammals, those found in cellular and intracellular membranous structures, are preferentially polyunsaturated, whereas depot or storage fat is predominantly saturated, possibly reflecting in part ergonomic considerations as more energy can be stored at a lower weight. The fat of wild game is roughly three times as polyunsaturated as that of commercial meat, reflecting the increased depot or storage fat which accumulates in domestic meat animals. In addition, game has more ω3 fatty acid (about 3% of total fat) than does commercial meat (about 0.4% of total fat in beef) (14).

Vegetable Foods

Millennia of agricultural practice have altered the fruits and vegetables most commonly consumed in Western nations. On average, the wild plant foods utilized by foragers provide more calcium (31), fiber (32), vitamin C (33) and vitamin E (Shostak, M., 1989, unpublished data) than do their cultivated counterparts. There have been changes in protein as well: for example, wild einkorn wheat has a greater protein content (22.8% crude protein) than does modern bread wheat (34), but most of today's raw fruits and vegetables still provide almost insignificantly small amounts of fat. Wild beans and nuts were probably the major sources of vegetable fat for our ancestors. Some cultures (*e.g.*, the Miwoks of California and the !Kung of Botswana) made nuts their major dietary staples, and most preagricultural peoples utilized them seasonally.

More important than the intrinsic fat content of individual plant items, which has changed relatively little over time, are the cultivation, storage, transportation and distribution capabilities of twentieth-century Western nations. Avocados (88% energy from fat), indigenous to North America, were inaccessible for all except Native Americans until the past few hundred years. Olives (90% energy from fat) and soybeans (47% energy from fat) are foods which had regional and seasonal importance for our ancestors but which are now consumed worldwide and year-round. They have assumed dietary significance for Mediterraneans and East Asians, respectively, which would have been impossible 10,000 years ago. Coconuts (92% energy from fat), one of the few plant foods whose lipids are overwhelmingly saturated, are now available everywhere, not just in Southeast Asia and the Pacific.

Paleontologically "New" Foods

Americans think of bread and milk as quintessentially "natural" foods. This is understandable, as nutritionists have designated cereal grains and dairy products as two of the four "essential" food groups. However, from the standpoint of genetically determined human biology, these foods are Johnny-come-latelies. No humans regularly consumed cereals until about 15,000 years ago, and the use of dairy products, apart from mother's milk, began later still.

Cereal grains. The potentially edible vegetation in tropical forests is almost exclusively dicotyledonous—fruits, shoots, buds, leaves, roots, etc.—so for 30 million years the prehuman primates in our ancestral line became genetically adapted to vegetable foods from this category. Existing great apes still focus on plant foods of this nature, so the present dependence of many human populations on monocotyledonous plants, especially cereal grains, is a notable departure from mainline primate experience, both in the past and currently (35).

Unprocessed cereal grains are poorly digested by the human gut; they must first be milled. Grindstones together with crude mortars and pestles designed for this purpose first appear in the archeological record during the final phase of the Late Paleolithic, perhaps 15,000 years ago in the Near East. Consequently, paleontologists infer that cereals constituted an insignificant dietary constituent for humans prior to that time (36).

Cereal grains present some intrinsic nutritional problems for humans (*e.g.*, their proportions of soluble and insoluble fiber, their calcium/phosphorus ratio, their limited calcium content, their phytate, etc.) (31,32), but although they do provide more fat than do most dicotyledonous plant foods, their lipid content seems relatively innocuous. Furthermore, traditional foods made from cereal grains, *i.e.*, breads, gruel, porridge, tortillas, etc., pose few, if any, lipid-related health problems for humans. However, many cereal-based bakery items popular in today's affluent nations represent significant sources of fat (Table 3).

Dairy products. Since they had no domesticated animals, Stone Age people had no dairy products whatsoever after they were weaned (typically around age three). The fat in mother's milk is valuable, if not essential, for normal postnatal development (*e.g.*, myelination), but for older children and adults dairy foods help increase dietary fat intake above the levels common for preagricultural humans. They do so when consumed as primary foods (*e.g.*, yogurt, ice cream) and also as complements (*e.g.*, toast and butter, cereal and cream) (Table 4). Furthermore, dairy fats are highly saturated, typically 55–65%, a proportion greater, on average, than that of any other commonly consumed food category except for tropical oils.

Food Processing and Preparation

The technology extant in industrialized nations has permitted profound distortion of the intrinsic nutritional properties of foods in several categories. Seed oils were

TABLE 3

Fat in Bakery Items[a]

	Fat, % total kcal
Biscuits	42.1
Pound cake	56.1
Chocolate chip cookies	52.5
Apple pie	39.0
Danish pastry	50.1
Raised doughnuts	57.9

[a]Source, see ref. 37.

TABLE 4

Dairy Products: Effects on Fat Intake[a]

	Total fat, g
Water, 8 oz.	0.0
Milk, 2% fat, 8 oz.	4.7
Coffee, black	0.0
With 1 tbs. light cream	2.9
Oatmeal, 1 cup	1.2
With 1/4 cup Half & Half	8.0
Whole wheat toast, 1 slice	0.7
With 1 tbs. butter	12.2

[a]Source, see ref. 37.

TABLE 5

Frying and Fat Content[a]

Item, 100 g	Total fat, g
Baked potato	0.1
French fried potato	39.6
Boiled onion	0.2
French fried onion rings	26.7
Corn, roasted	1.0
Corn chips	33.4
Shrimp, boiled	0.8
Shrimp, fried	10.8

[a]Sources, see refs. 22, 37 and 39.

not directly available until relatively recently, but now safflower, sunflower, rapeseed, corn and peanut oils form a significant part of many people's daily caloric intake. Stone Agers may have occasionally consumed some of the kernels and seeds from which such oils are obtained, but where the original foods provided fiber, protein and micronutrients, for practical purposes the processed oils provide only lipid. Except for those of tropical origin, most current vegetable oils are predominantly poly- or monounsaturated in composition. However, hydrogenation converts many of the unsaturated fatty acids to their saturated counterparts and, in addition, alters others to *trans* forms, where increasing evidence suggests they function more like saturated fats (38).

Our current propensity for frying foods gratuitously increases their fat content (Table 5). Humans have cooked their food for at least several hundred thousand years, and possibly for over a million (40), but while roasting, steaming and baking were all commonly employed techniques, frying was not. The necessary utensils were unknown and there was no ready source of fat for the purpose, at least in tropical climates where animals neither hibernate nor require insulation from excessive cold.

DIETARY COMPOSITION: PAST AND PRESENT

Late paleolithic humans. In 1985, the late Stone Age diet (of perhaps 20,000 years ago) was estimated to derive about 21% of its food energy from fat, and its polyunsaturated/saturated fatty acid ratio (P/S) was calculated to be about 1.41 (41). These estimates were derived from nutritional analyses of 21 wild game species and 44 uncultivated plant foods. The values for animals and plants were averaged separately and employed in a simple model which assumed a 35:65 animal/plant subsistence ratio (42) and a daily energy intake of 3000 kcal. (Other assumptions can easily be accommodated by this model.)

$$A(C^aX) + B(C^pX), \text{ assumed caloric intake} \quad [1]$$

Where A is mean energy content of animal food (kcal/g); B, mean energy content of plant food (kcal/g); C^a, proportion of animal food in diet; C^p, proportion of plant food in diet; X, total weight (g) of food necessary to provide an assumed caloric intake.

New information has become available since 1985, the data base now includes 41 species of game and 153 types of wild plant foods. For the animals, simple averaging was again employed; however, for vegetable resources a weighted average based on forager plant exploitation in eastern and southern Africa (43) was utilized. The resulting mean nutritional values for game and wild plant foods are given in Table 6. When substituted in the original model, they produce estimates of average paleolithic nutrition reasonably similar to those presented in 1985 (Table 7).

The increased fraction of daily energy derived from fat (24.7 *vs.* 21%) resulted chiefly from an increased number of seeds (16/153 *vs.* 1/44) in the expanded data base; the proportions of beans and nuts, and other relatively high-fat vegetal items, have remained fairly constant. As noted earlier, paleoanthropologists currently believe that substantial seed use by hunters and gatherers was uncommon until the very late Paleolithic Era (around 15,000 years ago), when a broad-based subsistence pattern became necessary (36,44). Accordingly, the updated projections for average wild plant fat content and for the total

TABLE 6

Nutritional Values for Game and Wild Plant Food

	Game		Wild plant food	
	1985 (n = 21)	1991 (n = 41)	1985 (n = 44)	1991 (n = 153)
Fat, g/100 g	3.8	4.2	2.84	3.8
Protein, g/100 g	24.3	22.7	4.13	5.2
Energy, kcal/100 g	140.8	132.7	128.76	149.0

TABLE 7

Estimated Average Daily Lipid Intake for Late Paleolithic Humans Consuming a 3000-kcal Diet

	1985	1991
Meat, g/d	788	732
Plant foods, g/d	1464	1360
Animal fat, g/d	29.7	30.7
Vegetable fat, g/d	41.6	51.7
Total fat, g/d	71.3	82.4
Fat, % total energy	21	24.7

TABLE 8

Fatty Acid Distribution in Average Paleolithic Diets

	Animal	Plant	Total	% Total fat
Saturated	11.4	8.1	19.5[a]	23.7[b]
Monounsaturated	11.1	23.4	34.5	42.0
Polyunsaturated	8.2	20.0	28.2[a]	34.3

[a]Polyunsaturated/saturated fatty acid ratio, 1.45.

[b]Saturated fat equaled approximately 6% of total energy intake.

contribution of fat to daily energy intake are probably overestimates for humans living prior to the last phase of the Paleolithic Era.

Complete fatty acid distribution patterns have been published for 17 wild game species (10,24,25). These, together with previously published estimates for fatty acid distributions in wild plant foods (41), suggest that the P/S ratio for late paleolithic diets was approximately 1.45 (the 1985 estimate was 1.41) (Table 8). The cholesterol concentration of meat from 14 wild animals averages 65.9 mg per 100 g portion, so paleolithic humans consuming 723 g of game each day would have had an average intake of 482 mg.

Current Americans. Recent estimates cited by the Committee on Diet and Health of the National Research Council (45) suggest that in the early 1980s Americans obtained 36–40% of their dietary energy from fat[2] (a range slightly below that of countries in northern and western Europe, ref. 46) and that saturated fats made up 38.5% of total fat intake. The P/S ratio was thought to be about 0.47. Cholesterol intake is estimated to have remained stable at approximately 480 mg/d between 1979 and 1985.

The Committee on Diet and Health, as do other expert groups in the United States, Canada and Europe, currently recommends that dietary fat be reduced to 30% or less of total energy intake, that saturated fatty acids supply less than 10% of total energy, that P/S should be approximately 1.0, and that dietary cholesterol average less than 300 mg per day. These authoritative recommendations can be compared with the estimated paleolithic nutritional pattern (Table 9). Of the differences, the most noteworthy probably involves dietary cholesterol intake. This discrepancy has potentially important economic implications; it will be comparatively easy for meat producers to reduce the fat content of animals raised for consumption, but decreasing tissue cholesterol concentration would be difficult (47).

TABLE 9

Paleolithic, Current and Currently Recommended Dietary Lipid Intake

	Paleolithic	Current	Recommended
Fat, % total energy	20–25	36–40	<30
Saturated fatty acids, % total energy	~6	15	<10
P/S ratio	~1.4	<0.5	~1.0
Cholesterol, mg/d	480	480	<300

[2]There is suggestive, although not conclusive, evidence that American fat consumption has been gradually declining over the past two decades (45).

SERUM CHOLESTEROL LEVELS

Americans, foragers and nonhuman primates. Just as generally accepted recommendations for lipid intake differ from estimated paleolithic human experience, norms for serum cholesterol concentration deviate from the observed levels in recently studied hunter-gatherer populations. The National Cholesterol Education Project (48) designates serum cholesterol levels below 200 mg/dL (5.18 mM/L) as "desirable." However, for five hunter-gatherer groups studied in this century, mean serum cholesterol concentration was 123.2 ± 7.2 mg/dL (33) (3.19 ± 0.19 mM/L). Furthermore, a larger series of pre-industrial populations, *e.g.*, 20 societies including foragers, rudimentary horticulturists, simple agriculturists and pastoralists exhibited an almost equally low mean level: males 131.8 ± 4.4 and females 135.2 ± 4.7 mg/dL (3.42 ± 0.11 and 3.50 ± 0.12 mM/L). Only three groups had mean values exceeding 150 mg/dL (3.89 mM/L), and none averaged as much as 175 mg/dL (4.53 mM/L) (49).

Free-living nonhuman primates have low serum cholesterol levels. For four species (Rhesus, Macaca and squirrel monkeys together with baboons), the average was 108.5 ± 11.9 mg/dL (2.81 ± 0.31 mM/L), and the highest mean value for any species was 135 mg/dL (3.50 mM/L) (50–53). The overlapping ranges of serum cholesterol values in foragers and nonhuman primates suggests that general primate physiology is designed to accommodate cholesterol levels of this magnitude, not those commonly found in modern Americans.

Serum cholesterol and coronary heart disease. American experience has shown that many individuals whose serum cholesterol levels are below 200 mg/dL nevertheless develop coronary heart disease, which is often sufficient to produce myocardial infarction. On the other hand, members of pre-industrial societies rarely show clinical manifestations of coronary atherosclerosis, and little or no significant coronary disease has been found when autopsy studies have been performed (49). Although many factors undoubtedly contribute to their reduced susceptibility, their low serum cholesterol levels are probably a major protective influence.

The understandably limited, near anecdotal evidence concerning coronary heart disease among hunter-gatherer populations has recently been supplemented by epidemiological studies among rural Chinese whose serum cholesterol levels are similar to those of foragers, *e.g.*, 126.6 ± 15.1 mg/dL (3.28 ± 0.39 mM/L) (54) and among whom mortality from coronary heart disease is less than one-tenth that in the United States.

Meat and serum cholesterol levels. Third World populations, like the rural Chinese, manifest "low" serum cholesterol levels although their diets include very little fat (15% of total energy intake) and relatively little protein (10% of total energy intake) (54). Saturated fatty acids comprise about 25% of total fat intake (4% of total energy intake) (55). It is perhaps significant that the cholesterol intake of rural Chinese (whose serum cholesterol levels are lower and who have less coronary atherosclerosis) exceeds that of urban Chinese (340 mg/d *vs.* less than 300 mg/d) (55).

Recently studied hunter-gatherers, who serve as "best approximation" surrogates for late Stone Age humans, achieve similarly "low" serum cholesterol concentrations although their subsistence is quite different. In some respects their lipid parameters are reasonably similar—dietary fat intake is 20–25% total energy and saturated fats contribute only about 6% of total energy intake. However, cholesterol intake, at 480 mg/d, is 50% greater and they consume approximately three times the protein, *i.e.*, 30% or more of total daily energy intake. Furthermore, most of this protein is of animal origin, about 70%.

The red meat commonly available to Western consumers includes considerable fat, and its consumption has been consistently correlated with elevated serum cholesterol levels. In contrast, the wild game available to recent hunter-gatherers, as to all preagricultural humans, was very lean. Current research has shown that such meat has little, if any, tendency to increase blood cholesterol levels, even when protein makes up a high proportion (24–30%) of total energy intake (56–58).

Simple agriculturists, like the rural Chinese, have probably consumed low-fat, low-animal protein diets for thousands of years, and it is appealing, therefore, to consider this nutritional pattern the human norm, especially since populations who eat in this way have little atherosclerosis and relatively few fat-related cancers (*e.g.*, breast, prostate, colon). However, even though a 5,000-year experience is impressive, it pales beside the 1,500,000-year period during which humans existed as hunters and gatherers consuming a low-fat but high-animal protein diet.

Propositions. These considerations logically imply a series of proposals for research, product development and, ultimately, nutritional and health guidelines: (i) The goal for dietary fat intake should be 20–25% of total energy intake, not 30%. (ii) The proportion of saturated fat needs to be decreased below 10% of energy intake. The P/S ratio should exceed 1.0, not as a result of increased polyunsaturated fat intake, but by marked reduction of saturated fat intake together with substitution of poly- and monounsaturated fat for saturated whenever possible. (iii) Cholesterol intake is a less significant dietary consideration and can moderately exceed 300 mg/d unless serum cholesterol levels remain undesirably high. (iv) The "desirable" level for serum cholesterol concentration is below 150 rather than below 200 mg/dL. Values below 150 mg/dL (~4 mM/L) are desirable, those between 150–200 (4–5 mM/L) are borderline, and those over 200 mg/dL (~5 mM/L) are dangerous. (v) The meat industry needs to devise economically viable methods of producing products whose nutritional properties duplicate those of wild game. (vi) Products based on milk and cereal grains need to be manipulated so that the beneficial properties of the raw materials are emphasized and their undesirable qualities, including their lipid content, are minimized.

The American food industry has made enormous contributions to human health by providing an ample, varied, safe and inexpensive food supply. It is now grappling with the interaction between diet and chronic illnesses. As the industry addresses this issue, it would do well to keep in mind our remote ancestors' likely subsistence pattern. Heart attack and breast cancer are commonly considered "diseases of civilization" because of their rarity among peoples whose eating habits maintain the essentials of our original human pattern. Their nutritional experience may well constitute a continuing paradigm for humans today.

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ARTICLE

Dietary Fat and Breast Cancer Risk: The Feasibility of a Clinical Trial of Breast Cancer Prevention[1]

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Animal experimental evidence and human ecological data suggest that dietary fat intake is related to breast cancer risk. Epidemiological studies within countries have given inconsistent results but are limited by the restricted range of dietary intake found in Western populations and by error in the measurement of fat consumption. Experimental evidence, derived from controlled clinical trials in which the range of fat intake is increased beyond that seen in most Western populations, is capable of overcoming this limitation of observational epidemiology, and would provide the strongest evidence available concerning the relationship of dietary fat intake to breast cancer risk. Further, such trials are the only means likely to answer the question of whether breast cancer risk in high-risk subjects can be modified by changing dietary fat intake. We describe here several aspects of the feasibility of an experimental approach to this problem, including the identification of subjects at increased risk for breast cancer, and the demonstration that such subjects will enter a clinical trial of dietary fat reduction and comply with a low-fat diet. It is shown that subjects can be recruited and retained in such trials, that satisfactory dietary compliance can be achieved over at least 24 mon and that the subjects selected are at demonstrably increased risk of breast cancer. This finding indicates that it is feasible to test the dietary fat-breast cancer hypothesis experimentally by means of a clinical trial.
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Breast cancer is the commonest cancer in women in most of the developed world. It is the commonest cause of death from cancer among women at all ages and the commonest cause of death from any cause in women aged less than 50 (1). Mortality from breast cancer has not changed over a substantial period of time, despite intensive research in methods of treatment, and the best prospects for reducing mortality from this disease at present lie in screening and prevention.

There is considerable evidence that breast cancer is, in principle, preventable (2). This evidence comes from the sevenfold variation in incidence of the disease seen internationally, from increasing breast cancer incidence in migrants from low-risk to high-risk countries, who eventually acquire the risk of their adoptive country, and from the increase in the incidence of the disease that has been observed in several formerly low-risk countries. The identity of the factors responsible for the influence of environment on breast cancer incidence is at present unknown, but evidence from animal experiment and human epidemiological data suggests that diet, and in particular dietary fat, may play a key role. This paper briefly discusses the evidence that dietary fat intake may be related to breast cancer risk and describes the rationale, methods and early results of a clinical trial designed to test experimentally the dietary fat breast cancer hypothesis.

Evidence that fat intake is related to breast cancer risk. Dietary fat intake influences breast cancer risk in animals (see refs. 3-5 for recent reviews). In animals, increasing intake of dietary fat increases tumor incidence, increases the number of tumors that develop per animal and decreases the latent interval before the appearance of tumors. When given with a carcinogen, dietary fat acts as a tumor promoter and appears to have an effect on tumorigenesis that is independent of caloric intake.

Human ecological studies comparing breast cancer incidence or mortality with dietary fat consumption within countries show a more than fivefold variation in breast cancer rates between countries which is strongly correlated (r = 0.8-0.9) with international variation in estimated dietary fat intake. Countries with the highest estimated fat intake in general also have the highest breast cancer rates, and countries with the lowest fat intake the lowest rates. Prentice *et al.* (6) have examined international breast cancer rates and estimates of fat consumption using regression analysis. Dietary fat intake explained more of the international variation in breast cancer (58%) than did total calories (14%) or any other dietary constituent considered and the effect of fat remained highly significant after controlling for differences in intake of total calories between countries. Dietary fat remained significantly associated with breast cancer rates after controlling for Gross National Product, height, weight and age at menarche (6). These analyses therefore showed a remarkably consistent effect of dietary fat intake in explaining international differences in the frequency of breast cancer.

Rationale for a clinical trial. Observational cohort and case control studies, however, have given much less consistent results (see refs. 7-12 for recent reviews). The largest of these is the Nurses Health Study (13) which showed no association between dietary fat intake and breast cancer risk in more than 100,000 nurses in the United States followed for 6 yr. However, it is now recognized that the intake of dietary fat in "western" countries is remarkably homogeneous (6,9,10). Given the narrow range of dietary fat intake observed in the Nurses Health Study and the error known to be associated with the method used to measure fat consumption in that study, it is unlikely that the study would have found an association between fat intake and breast cancer risk, even if

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Abbreviations: BCDDP, Breast Cancer Detection and Demonstration Projects; CI, confidence interval; HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein.

international variation in breast cancer rates is entirely due to differences in fat intake. Prentice *et al.* (6) calculated that the Nurses Health Study had only 24% power to detect the 15% gradient in breast cancer risk across the observed gradient in fat intake that would be expected from international ecological data. A recent reanalysis of twelve case control studies from several countries by Howe *et al.* (14) showed, in contrast to the Nurses Health Study, a strong evidence of an association between dietary fat intake and breast cancer risk, strongest in postmenopausal women. Observational epidemiological studies, which are the conventional approach taken to examine potential etiological associations, are severely constrained in their ability to answer questions about the relationship of dietary fat intake to breast cancer risk in humans.

Experimental evidence, derived from controlled clinical trials in which the range of fat intake is increased beyond that seen in most Western populations, is capable of overcoming this limitation of observational epidemiology, and would provide the strongest evidence available concerning the relationship of dietary fat intake to breast cancer risk. Further, such trials are the only means likely to answer the question of whether breast cancer risk in high-risk subjects can be modified by changing dietary fat intake.

We describe here several aspects of the feasibility of an experimental approach to this problem, including the identification of subjects at increased risk for breast cancer and the demonstration that such subjects will enter a clinical trial of dietary fat reduction and comply with a low-fat diet.

METHODS

Selection of subjects. Subjects were recruited from the Breast Diagnostic Clinics at Women's College Hospital, Mount Sinai Hospital, St. Michael's Hospital and the Toronto Western Hospital between 1982 and 1990. Subjects were eligible for the trial if they were aged at least 30 yr and had been examined by mammography within 3 mon of entry and found to have at least 50% of the breast volume occupied by the radiological changes of dysplasia. Subjects were excluded if they were pregnant or breast feeding, if they were taking a medically prescribed diet for any reason, or if they had a previous history of breast cancer. Potentially eligible subjects were identified first from lists prepared in the Departments of Radiology at collaborating hospitals where mammograms were classified according to the extent of mammographic dysplasia, and were then contacted by letter from their referring surgeon. The letter described the study in outline, was accompanied by a brochure giving the background and procedures involved in the study and indicated that one of the study personnel would telephone the subject within a few days. The purpose of the telephone call was to answer any questions concerning the study, to identify those subjects interested in entering the study and to arrange an appointment for them with a study dietician.

Preliminary assessment. After an appointment with a dietician had been arranged, subjects were enrolled in the study in two phases using different procedures. These phases involved the same events but differed in the sequence in which they occurred and in the time required for their completion. In Phase I, used initially, dieticians explained and discussed the study with subjects and sought signed consent. Subjects who provided consent were then allocated randomly to control or intervention groups. For subjects in the intervention group, dietary counselling and the preparation of an individual dietary prescription then began (see below) and the subject was seen monthly for the following 12 mon.

In Phase II, the procedure for entry adopted subsequently, the dietician at the first visit explained the study as before but then taught the subject to keep food records, using scales and household measures to record quantitatively all food consumed during a specified 3 d including one weekend day. At a second appointment, these records were reviewed and for subjects who had completed records of satisfactory quality, consent was then sought and randomization carried out. The quality of the food records was assessed subjectively by inspecting them for the completeness and the amount of quantitative detail required for their subsequent coding and nutrient analysis. All dieticians working in the trial had been certified by the Nutrition Coordinating Center and were familiar with the procedures of coding and analysis. Dietary counselling and the preparation of an individual diet prescription then proceeded as before. For subjects who failed to keep satisfactory records, further instruction was given and record keeping repeated. Consent for entry to the study was only sought after records of adequate quality had been kept.

Randomization and dietary intervention. For dietary intervention studies, subjects were randomly allocated to either a control group who were taught the principles of a healthy diet using Canada's Food Guide but who were not counselled to change the fat content of their diet or to an intervention group who were taught how to reduce their dietary fat intake to an average target level of 15% of calories. This was done by preparing an individualized dietary prescription, based on a detailed assessment of subject's eating habits at entry to the study. In this prescription, fat was substituted by the isocaloric exchange of complex carbohydrate. Subjects were encouraged to introduce these dietary principles into their diet as soon as possible and to adopt the new diet fully within 4 wk. In addition, we provided several dietary aids that included dietetic scales, a food guide containing the subject's individualized meal pattern, daily food allowance and additional information such as exchange lists for fat, cereals, fruit and vegetables, an extensive shopper's guide, suggestions for eating away from home and approximately 200 low-fat, high carbohydrate recipes.

For follow up during the trials and for assessment and promotion of compliance, subjects in the intervention group were seen once every month for 12 mon, and those in the control group once every 4 mon for 12 mon. At each visit, both groups provided a record of foods eaten on three randomly selected days and a 1-d dietary recall. Nutrient intake as indicated by these sources was initially calculated manually and compared with the diet prescription to assess compliance, to provide the subjects with feedback and to serve as the basis for discussion about difficulties or about further changes that might be made. Particular attention was paid at follow-up visits to the maintenance of caloric intake and preservation of initial weight. Food records were subsequently analyzed by the Nutrition Coordinating Center, Minneapolis, MN, and the results of these analyses are shown in the present paper.

RESULTS

Characteristics of subjects. In Phase I of the study, 227 subjects were randomized, after providing consent but before being taught to keep food records. Their characteristics have been described elsewhere (15). Subjects in the control and intervention groups were similar with respect to demographic features and risk factors for breast cancer. The mean age of subjects was 44 yr and 75% were premenopausal at entry. Approximately 20% of subjects had a first-degree relative with breast cancer, 39% of the intervention group and 40% of controls were nulliparous and the mean age at first live birth of those who were parous was 25 yr. The characteristics of subjects entering Phase II, which is still in progress, are shown in Table 1. The mean age, the proportion who are premenopausal and the high prevalence of first-degree relatives with breast cancer are similar to Phase I. Intervention and control groups again resemble each other with respect to risk factors for breast cancer.

Recruitment and retention of subjects. In Phase I, following randomization, of 115 subjects allocated to the control group, 99 (86%) remained in the study at the end of 12 mon compared to 80 (71%) of the 112 subjects allocated to the intervention group. Statistical comparison of the proportion of subjects remaining in the control and intervention groups showed that they differed significantly ($P = 0.011$). Of a total of 32 dropouts from the intervention group, 19 (59%) occurred within 60 d of randomization. Of 16 dropouts from the control group, 11 (69%) occurred within 60 d of randomization. Many of those who dropped out did so at randomization and never returned for further appointments and so provided no nutrient data.

In Phase II, a total of 25 (9%) subjects dropped out, 14 from the intervention group and 11 controls. Approximately 90% of both the intervention and control groups showed no significant differences ($P = 0.31$). The probability of subjects in the intervention groups in Phases I and II of the study remaining did differ significantly ($P = 0.0001$). The probability of controls remaining in the study for 12 mon was not significantly different in Phases I and II ($P = 0.13$).

Nutrient intake. The intake of dietary fat as assessed by the nutrient analysis of food records at baseline, 4, 8, and 12 mon after randomization in Phase I has been reported elsewhere (15,16). Results to date for Phase II are shown in Table 2. The intake of dietary fat in the control group, in terms of the proportion of calories derived from fat, remained stable over the 24 mon of observation. Four months after randomization, the dietary fat intake of the intervention group fell from a mean of 33% of calories at baseline to a mean of 22%. From 4 mon to 24 mon after randomization, approximately 60% of the intervention group had an intake of dietary fat that fell within 5% of the target of 15% of calories, and approximately 80% had an intake within 10% of this target. Protein intake was unchanged and the mean percentage of calories from carbohydrate rose from 43% to 56%. The reduction in total fat intake in the intervention group was due to a reduction in both saturated and polyunsaturated fat, and the ratio of these sources of fat did not change over the course of 24 mon. No change in dietary fat intake was seen in the control group. Intake of dietary cholesterol also fell in the intervention group. The increase in carbohydrate consumption after randomization shown in Table 2 was attributable to increased intake of starch and other complex carbohydrates without any significant increase in sucrose consumption. No change in carbohydrate intake was seen in the control group.

Statistical comparison of fat and carbohydrate intake between intervention and control groups after randomization was highly significant ($P < 0.0001$) at all intervals after randomization. The changes in dietary fat consumption indicated in the food records were supported by a quantitative relationship between nutrient intake and changes in serum cholesterol (see below).

Association of nutrient intake and serum cholesterol. Duplicates of all food consumed during one 24-h period were collected from 57 volunteer subjects, 29 from the intervention group and 28 from the control group, at either 6 or 12 mon after randomization, and foods homogenized and chemically analyzed. Chemical analysis of fat showed that the intervention group consumed an average of 27 g per day (18% of calories) and that the control group consumed an average of 47 g per day (31% of calories) (16).

Further, the nutrient intake recorded in the food records was supported by a quantitative relationship between changes in intake and changes in serum cholesterol. Serum cholesterol in women in the intervention group fell an average of 8% at 4 mon, 6% at 8 mon and 4% at 12 mon. Predicted changes in serum cholesterol were calculated by the formulas of Keys and Hegsted. Observed changes were not significantly different from the changes predicted for subjects with initial serum cholesterol values in the upper tertile of the population in whom serum cholesterol fell 14% at 4 mon, 12% at 8 mon and 10% at 12 mon. Observed changes were also not significantly different from those predicted for subjects with baseline values in the middle tertile but were significantly less than predicted for those with initial values in the lower tertile in whom values rose 3% at 12 mon. Regression analysis now showed that the prediction of change in serum cholesterol for a given change in diet can be substantially improved by a model that includes initial serum cholesterol value and

TABLE 1

Distribution of Selected Characteristics in Subjects in Intervention and Control Groups (present study)

Characteristics	Intervention	Control
Number of subjects	249	248
Mean age in years	44	45
Marital status (%)		
Never married	22	19
Divorced	9	9
Married	63	68
Separated	4	3
Widowed	2	2
Mean weight in kg	61	62
Mean height in cm	163	163
Mean age at menarche	13	13
Parity (% parous)	61	60
Mean age at first live birth (years)	25	26
First-degree relative with breast cancer (%)	21	19
Menopausal status (%):		
Premenopausal	79	77
Postmenopausal	21	23

TABLE 2

Intake of Selected Nutrients as Assessed by Food Records According to Group and Time of Data Collection

		Time											
		0			4			8			12			24		
Nutrient	Group	Mean	STD	N	Mean	STD	N	Mean	STD	N	Mean	STD	N	Mean	STD	N
Energy, kcal	Intervention	1592.8	414.7	222	1433.3	388.9	179	1476.9	377.4	149	1481.9	363.7	110	1530.0	445.3	36
	Control	1639.4	458.4	219	1650.9	459.4	93	1614.4	472.8	65	1629.8	431.7	127	1642.5	347.3	35
Total fat (%)	Intervention	32.7	7.1	222	2.0	6.1	179	22.3	7.0	149	22.1	6.9	110	22.8	7.3	36
	Control	33.2	6.5	219	34.0	6.4	93	33.2	6.9	65	32.6	7.7	127	33.6	6.4	35
Saturated fats (%)	Intervention	12.1	3.4	222	7.4	2.5	179	7.5	2.8	149	7.4	2.8	110	7.6	2.9	36
	Control	12.0	3.5	219	12.8	3.3	93	12.6	3.4	65	12.1	3.6	127	12.3	2.9	35
Polyunsaturated fats (%)	Intervention	6.3	2.3	222	4.6	1.6	179	4.7	1.9	149	4.6	1.7	110	5.1	2.4	36
	Control	6.6	2.1	219	6.4	2.1	93	6.3	2.0	65	5.9	1.9	127	6.7	2.3	35
Monounsaturated fats (%)	Intervention	11.7	3.1	222	7.7	2.5	179	7.8	2.9	149	7.8	2.9	110	7.8	2.9	36
	Control	11.9	2.7	219	12.1	2.8	93	11.8	2.7	65	11.9	3.6	127	11.9	2.7	35
Protein (%)	Intervention	17.5	3.8	222	18.4	3.7	179	18.2	3.6	149	18.3	3.4	110	18.7	2.7	36
	Control	17.2	3.5	219	17.2	3.3	93	17.7	3.7	65	17.8	3.4	127	17.6	3.6	35
Total carbohydrates (%)	Intervention	48.6	8.3	222	59.0	8.2	179	58.4	9.6	149	58.6	9.5	110	58.9	9.3	36
	Control	48.2	8.5	219	47.8	7.5	93	48.3	8.0	65	49.2	8.9	127	46.5	8.0	35
Cholesterol (mg)	Intervention	255.8	125.8	222	184.2	92.5	179	184.9	94.2	149	182.8	96.5	110	216.3	128.9	36
	Control	263.1	132.2	219	265.4	111.5	93	263.4	119.5	65	252.6	127.9	127	263.7	100.9	35
Alcohol (%)	Intervention	3.2	3.9	222	3.0	3.8	179	3.3	4.4	149	3.3	4.5	110	1.9	3.2	36
	Control	3.3	4.6	219	3.0	3.9	93	2.5	3.6	65	2.3	3.4	127	4.1	4.7	35

change in total fat intake ($R^2 = 0.41$) than by the Keys or Hegsted formulas ($R^2 = 0.04$), which do not include initial serum cholesterol value. Details of the relationship between changes in nutrients and serum cholesterol have been given elsewhere (17).

Cancer risk. Seventeen invasive cancers have been found to date, 5.2 times the number expected (95% confidence interval [CI] 2.9–8.4) based upon age-specific person years of follow-up for the Ontario population. This increase in risk persists after exclusion of cancers found within 12 mon of randomization (risk = 5.2 times population risk; 95% CI = 2.8–8.9). We can therefore be confident that the risk observed in this trial population is substantially greater than the risk in the general population and that a group at high risk for breast cancer is being selected.

Sample size required for a breast cancer prevention trial. The sample size for a trial is based upon the expectation that cancer incidence will be reduced by 35% in the intervention group. As is discussed below, the planned duration of this trial is 10 yr which will comprise 2 yr of patient entry and 8 yr of follow-up. The sample size will be sufficient to provide an 81% probability of detecting an effect of this size.

Estimation of cancer incidence in the control group. We have estimated the cancer incidence of the control group from data reported from the Breast Cancer Detection and Demonstration Projects (BCDDP) (18). The report of Brisson *et al.* (18) describes the breast cancer incidence over 9 yr of observation in women with "dense or glandular" breast parenchyma at entry (an appearance of the breast parenchyma that corresponds approximately to the appearance of extensive dysplasia that will be used to select subjects for the present trial). This report shows that there is an elevation of breast cancer risk in women with dense breast parenchyma at entry, relative to women without dense breasts, that persists for at least 9 yr. The incidence of breast cancer according to age at entry is shown in Table 3. Age-specific cancer risks were applied

TABLE 3

Distribution of Subjects According to Age and Expected Annual Cancer Incidence in Proposed Clinical Trial

Age at entry	CI[a]	Annual rate	Proportion[b]
35–49[c]	16.04	2.01	0.77
50–54	25.55	3.19	0.143
55–59	26.45	3.31	0.067
60–65	54.78	6.85	0.022

[a] CI, cumulative incidence of breast cancer per 1,000 over years 2 to 9. The data are taken from Table 1 of reference 20 supplemented by information kindly supplied by Dr. Brisson. These rates have been used to calculate an average annual incidence (3rd column) from which 10 yr incidence has been estimated.

[b] This column shows the proportion of subjects according to age group in our present trial.

[c] The distribution of subjects aged 35–49 in 5-yr age groups within the range 35–49 years is similar in the BCDDP and in our present population. In both populations approximately equal proportions of subjects are within each of these 5-yr age groups. In our population 0.28, 0.26, and 0.23 of all subjects are respectively in the 5-yr age groups 35–39, 40–44, 45–49. In the BCDDP 0.14, 0.17, and 0.18 of all subjects were in these 5-yr age groups. We have therefore treated the age group 35–49 as a single category for the purposes of risk calculations.

to a population with the age distribution observed in the 600 subjects enrolled in our trials to date to derive an expected breast cancer incidence in the absence of the intervention.

Estimation of the effect of the intervention. The risk reduction has been assumed to be linear and to decline from 1.0 at the start of the trial to a final relative risk of 0.30 at the end of 10 yr. This estimate of the effect of the intervention is derived from estimates of the effect of dietary fat on breast cancer risk from international epidemiological data and from the threefold or greater changes in risk that have been observed in migrants (6). Because of the age of the population, competing causes of death should be negligible and have been ignored in the calculations.

Sample size required. Using an adaptation of the procedure described by Self *et al.* (19), taking into account the age distribution of the subjects already enrolled and assuming a linear decline in risk, we calculate that 8,500 subjects recruited over 2 yr and followed for 8 yr will give an 80% probability of detecting a relative risk of 0.3 at the end of 10 yr. Because we expect a drop-out rate of approximately 4% in the first year after entry and 1% per year thereafter, we need to recruit a total of 9,500 subjects to ensure that 8,400 will remain in the study at the end of 10 yr. We expect 105 cancers in the control group and 68 in the intervention group for a reduction in breast cancer incidence of 35%. (Drop-outs will also be followed for the development of cancer but are not included in these calculations.)

The risk of breast cancer in the control group may, however, be higher than estimated. Quantitative methods of classifying breast dysplasia, and standardized readers will be used rather than the qualitative methods of classification used in the BCDDP. Quantitative methods of classifying breast densities have, in general, identified groups of subjects with higher risks of breast cancer than have qualitative methods (20–22), and while 5% of subjects in BCDDP with dense breasts had first degree relatives with breast cancer (23), 20% of subjects recruited to date have at least one first-degree relative with breast cancer. Further, the observed cancer risk in subjects enrolled to date is approximately double that estimated from BCDDP data, even after the exclusion of cancers diagnosed within 12 mon of entry.

Influence of recruitment from breast screening centers on trial power. The Ontario Provincial Breast Screening program plans to examine with mammography all women in the population over age 50. Recruitment to the trial from screening centers will change the age distribution of the trial population from that used above to calculate sample size to one with a higher mean age. We have examined the potential effect of this change on the power of the trial by recalculating age-specific incidence rates for breast cancer using the age distribution expected in the screening program. We would expect 184 cancers in the control group. A trial of this size and age distribution could detect with 80% power a 27% reduction in the incidence of breast cancer over a period of 10 yr, when we would expect 136 cancers in the intervention group. A 27% reduction in breast cancer incidence would be associated with a final relative risk of 0.44, considerably more conservative than the final relative risk of 0.3 on which calculations of sample size were originally based. The inclusion of subjects recruited from breast screening centers will therefore increase the ability of the trial to detect an effect of dietary fat reduction on breast cancer incidence.

DISCUSSION

Animal and human ecological data suggest that dietary fat may play a major role in the aetiology of breast cancer. Human epidemiological evidence is, however, inconsistent, and because of the homogeneity of dietary fat intake in most populations combined with the substantial error associated with measurement of fat intake in most epidemiological studies, but this does not exclude a strong association between fat consumption and disease risk. Evidence now exists indicating that the experimental study of the influence of dietary fat reduction on breast cancer incidence is feasible in a randomized controlled trial. Feasibility has been shown for the recruitment and retention of subjects, for dietary compliance and for the observed cancer risk of the subjects selected. Further, the sample size required for such a trial is attainable in Canada by a multicenter trial. Such a trial would provide the strongest evidence available concerning the relationship of dietary fat intake to breast cancer risk and is the only means to determine whether high-risk subjects can reduce their risk by a modification in diet.

Several unanswered questions remain, however. These include the feasibility of maintaining the dietary intervention past the present period of observation of 24 mon and the uncertainty as to whether the control group will not change their diets and come to resemble more closely the intervention group. A substantial reduction in the difference in fat intake between intervention and control groups would of course greatly reduce the ability of a trial to detect any influence of dietary change on breast cancer incidence. A longer period of observation will be required before these questions can be answered with certainty. However, we have to date not observed any tendency for compliance in the intervention group to diminish over time, and follow up data (not shown here) from subjects enrolled in Phase I for 1 yr shows persistence of dietary modification after the cessation of dietary counselling. It appears therefore that a sustained period of dietary change may give rise to long lasting changes in eating patterns. Similarly no evidence has been detected to date of dietary fat reduction among control subjects.

The randomized trial described here thus provides opportunities to examine a number of potential mechanisms for the postulated effect of dietary fat on breast cancer risk. These include the effects of diet on plasma lipids, other products of fat metabolism and plasma sex hormones.

Plasma lipids have been extensively studied in the context of ischaemic heart disease, and there is now also evidence that lipids, particularly high density lipoprotein cholesterol (HDL-C) may be related to breast cancer risk (24–26). In short term human experimental studies, it has been shown that reduction in dietary fat intake of the magnitude proposed here is followed by a fall in plasma levels of HDL-C (27–30). These studies and preliminary data from previous work of our own (16) show that in women low-fat high-carbohydrate diets, including the current diet recommended by the American Heart

Association, reduce plasma HDL-C by approximately 8%. Total serum cholesterol and low-density lipoprotein cholesterol fall by about the same amount. All studies to date have been of short duration, our own to 12 mon being the longest. Because HDL is a strong predictor of risk of ischaemic heart disease in women, there is a need to document the longer term effects of dietary fat reduction on all blood lipids and lipoproteins (31).

Reduced-fat diets have been shown to lower serum estradiol in postmenopausal women with breast cancer (32), premenopausal women with cystic disease (33) and healthy postmenopausal women (34). Plasma estradiol levels have also been found to be lower in premenopausal vegetarians than in omnivores (35). All of these studies have been of short duration, usually a few weeks, and to our knowledge the longer term effects of dietary fat reduction on plasma hormone levels have never been studied despite the widespread belief that both estradiol and dietary fat may be relevant to carcinogenesis in the breast.

The trial thus provides a setting in which answers to these and other biological questions relevant to both cancer and heart disease can be addressed and the relationship of these and other potential intermediaries of disease risk can be examined in relation to the relevant disease outcomes.

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ARTICLE

Utilization of Extracellular Lipids by HT29/219 Cancer Cells in Culture

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Uptake and incorporation of long-chain fatty acids were studied in a human colorectal cancer cell line (HT29/219) grown in culture medium supplemented with either fetal calf serum (FCS) or horse serum (HS). The cells were grown for 120 h with no change of medium; the two major cellular lipid classes, the phospholipids and the triacylglycerols, were analyzed at regular time-points. We observed significant changes in the concentration of most fatty acids throughout culture, and differences in their composition when different sera were used to supplement the medium. Minimal levels of free fatty acids were found in the cells, indicating a very small "free fatty acid pool". A major difference between the cells grown in media supplemented with different sera was the changes observed in concentrations of cellular polyunsaturated fatty acids during growth. In cells grown with FCS (in which 20:4n-6 is present), the levels of this acid in the phospholipid and triacylglycerol fractions declined rapidly during cell growth, suggesting further metabolism. In cells grown in medium supplemented with HS, 18:2n-6 was the major polyunsaturated acid present. There was clear evidence that this acid accumulated in the cellular triacylglycerol and phospholipid fractions. Furthermore, its concentration did not decline during growth in culture, suggesting minimal conversion to other polyunsaturated n-6 acids. Our results suggest that fatty acids from additional sources in the medium, for example triacylglycerols and phospholipids associated with the lipoproteins, are taken up by the cells. There is also indication of cellular fatty acid synthesis, particularly of monounsaturated and saturated acids during the culture period. HT29/219 cells were shown to take up and incorporate radioactivity when trace amounts of [1-^{14}C]-labeled arachidonic, linoleic or oleic acids were added to the culture medium. Most (80%) of the label was detected in cellular phospholipids and triacylglycerols, although the specific activities of these various fatty acids were different in the two lipid fractions.

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To compare data from different laboratories studying lipid metabolism in cultured cells, growth conditions must be kept as constant as possible. It has been shown that the serum supplement is important in studies on the lipid metabolism of cultured cells, as it is the most prevalent source of lipids in culture media. One of the major lipid fractions in serum, if not the main fraction, taken up by cultured cells, are free fatty acids (1–4). Cells grown in media supplemented with different sera (which contain different types and concentrations of free fatty acids) are thus subjected to different lipid environments. Investigations involving modification of cellular lipids, in particular the phospholipids in the membrane, have shown that the cell membrane's normal barrier function, as well as the responsiveness of the integral proteins, are affected when cellular fatty acid levels and compositions are changed (5,6).

The present study was designed to investigate the effect on cellular fatty acid levels when a cancer cell line was grown in media supplemented with different sera. Two serum supplements containing different concentrations of fatty acids were used. We have analyzed the fatty acid composition of the different lipid classes in a human colorectal carcinoma line grown in fetal calf serum (FCS)- supplemented and horse serum (HS)-supplemented media. FCS and HS, which are the two most commonly used serum supplements in cell culture studies, were first analyzed to obtain detailed information about their fatty acid composition.

Although in the routine culture of the cells used in this study, the medium is changed and/or the cells are passaged once every 48 h, for the purposes of this work no medium change was carried out during the period of culture (120 h). The reason for this approach was that we were particularly interested in studying fatty acid uptake and turnover in the cells during the rapid logarithmic phase of growth. From preliminary studies using HT29/219 cells grown in fetal calf serum-supplemented medium, 120 h was shown to be a satisfactory time period as cells were found to grow most rapidly between 48 and 96 h.

MATERIALS AND METHODS

Materials. HT29/219, a human colorectal carcinoma cell line, was obtained from the European Animal Cell Culture Collection (Porton Down, Salisbury, U.K.). Dulbecco's modification of Eagle's essential medium (DMEM) was purchased from Flow Laboratories Ltd. (Rickmansworth, U.K.). FCS, HS, trypsin, penicillin and streptomycin were from Gibco-BRL Ltd. (Irvine, Scotland). Ethylenediaminetetraacetic acid (EDTA) was supplied by BDH Ltd. (Glasgow, U.K.). Brain heart infusion (BHI) and Sabouraud liquid medium (SAB) were from Oxoid (Basingstoke, U.K.). Fatty acid standards were obtained from Sigma Chemical Co. Ltd. (Poole, U.K.), and [1-^{14}C]-labeled acids were from Amersham International (Aylesbury, U.K.). All solvents used were of high-performance liquid chromatography (HPLC) grade and supplied by BDH Ltd.

Cells and culture conditions. Stock cultures of cells were maintained in 75-cm^3 flasks containing 30 mL of DMEM supplemented with 10% fetal calf serum, penicillin (50 IU/mL) and streptomycin (50 μg/mL) at 37°C in a humid-

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Abbreviations: BHI, brain heart infusion; BHT, butylated hydroxytoluene; DFC10, DMEM enriched with 10% fetal calf serum (vol/vol); DH10, DMEM enriched with 10% horse serum (vol/vol); DMEM, Dulbecco's modification of Eagle's essential medium; EDTA, ethylenediaminetetraacetic acid; FAME, fatty acid methyl esters; FCS, fetal calf serum; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; HS, horse serum; PBS, phosphate buffered saline; SAB, Sabouraud liquid medium; TLC, thin-layer chromatography.

ified atmosphere of 5% CO_2/95% air. This medium will be referred to as DFC10. To study the effect of horse serum on cellular lipid metabolism, the cells were subcultured in DMEM supplemented with 10% HS for a minimum of five passages prior to the setting up of the experiments. This medium will be referred to as DH10. Cultures were periodically monitored for bacterial and fungal contamination using BHI and SAB, respectively. Viability was assessed by Trypan blue exclusion and was determined to be >93% for all experiments.

Cells grown routinely (either in DFC10 or DH10) were harvested *via* trypsinization. The detached cells were washed with and then suspended in the medium to be used for their growth. Cells were seeded at a density of 1.9×10^4 cells/cm^2 in 9-cm diameter petri dishes and grown in 10 mL of either DFC10 or DH10 for up to 120 h. After incubation for specific time periods (4, 24, 48, 72, 96 and 120 h) at 37°C, plates were removed from the incubator and the medium removed and kept at −20°C for lipid analysis. In our study, the 4-h time-point was regarded as representing the initial state of the cells. No analyses were carried out at the true start of the experiments (0 h) because the HT29/219 cell line is an anchorage-dependent cell line. As the cells had not attached to the plates until at least 4 h after plating, studying their growth and metabolism at any time before 4 h was not a true starting point for comparison with other time-points.

The attached cells were washed twice with approximately 1 mL of ice-cold phosphate buffered saline (PBS) solution and harvested by scraping with a rubber policeman. After centrifugation, the cell pellets were stored at −20°C until further analysis (see below).

Lipid extraction and analysis. The cell pellets were resuspended in 1 mL of PBS and disrupted by three freeze-thaw cycles followed by sonication using an ultrasonicator. An aliquot of the cell suspension was removed for protein analysis by a turbidimetric method (7). This method correlated well with the more traditional Lowry method (8) of protein determination and was preferred to the Lowry method due to the reported unspecificity of the latter (9). Total lipids were extracted from the remainder of the cell suspension according to the procedure of Folch *et al.* (10) using chloroform/methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. The lipids were fractionated into phospholipids, cholesterol, triacylglycerols and free fatty acids by thin-layer chromatography (TLC) on 0.25-mm Silica Gel 60 precoated plates (Merck, Darmstadt, Germany) using hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) as the developing solvent. Dichlorofluorescein (0.1%) in 95% methanol was used to spray the plates, and the bands were visualized under ultraviolet light. The phospholipid, triacylglycerol and free fatty acid bands were scraped off and the lipids eluted with chloroform/methanol (2:1, vol/vol) containing 0.01% BHT. At this stage of the analysis, heptadecanoic acid methyl ester (5 μg/mL of cell suspension for early time-points and 50 μg/mL for later time-points) was added to each extract as an internal standard (11). Phospholipids and triacylglycerols were transesterified using sodium methoxide-methanol (12), and free fatty acids were methylated using diazomethane (13). The fatty acid methyl esters (FAME) were dissolved in dry heptane and analyzed on a Hewlett-Packard benchtop gas chromatography/mass spectrometry (GC/MS) system (Winnesh, U.K.) equipped with a 30-m DB-WAX column with an internal diameter of 0.25 mm and 0.25 μm film thickness (J&W Scientific, purchased from Jones chromatography Ltd., Mid Glamorgan, U.K.). Details of the operating conditions and quantitative analysis of the FAME are published elsewhere (14).

The recovery of triacylglycerols and free fatty acids throughout the workup was found to be 95% ± 9% and 84% ± 14%, respectively. For phospholipids the recovery was lower (42% ± 4%). However, all data presented have been corrected for these losses.

Free fatty acids were extracted from an aliquot of the culture medium at each time-point, as well as at the start of the experiments, using chloroform/heptane/methanol (28:21:1, vol/vol/vol) containing 0.01% BHT (15). Heptadecanoic acid methyl ester (5 μg/mL of culture medium) was used as an internal standard, and the dried extract was methylated using diazomethane (see above). The recovery of free fatty acids in this procedure was found to be 92% ± 7%. The results presented have been corrected accordingly.

Incubation of cells with radiolabeled fatty acids. In order to investigate the uptake and incorporation of free fatty acids into cellular lipids, [1-^{14}C]arachidonic, [1-^{14}C] linoleic or [1-^{14}C]oleic acid (15 nCi/mL) were added in ethanol to the cell suspension at the time of plating. The final concentration of [1-^{14}C]-labeled acid and ethanol were 75 nM (equivalent to 150 nCi/plate) and 0.05% per plate, respectively. Cells were incubated as above, harvested at each time-point, lysed, and the lipids extracted and fractionated as outlined above. The separated lipids were then scraped off the TLC plates for counting of radioactivity. A portion of the growth medium was also removed at each time-point for measurement of radioactivity remaining in the medium.

Statistical methods. For experiments where three or more replicates were available, means, standard deviations and coefficients of variation were calculated in the usual way. The 95% confidence limits also were determined using the appropriate t values from the t-distribution table. Statistical significance was tested by the Mann-Whitney U-test.

RESULTS

HT29/219 cells were found to grow in medium supplemented with either FCS or HS. The growth rate of the cells, measured as the change in the amount of protein with time, was, however, found to be higher in DFC10-grown cells after 48 h (Fig. 1). Fatty acid analysis of the two serum supplements revealed significant differences in both the concentrations of individual lipids and the types of fatty acids present. Table 1 shows the concentration of fatty acids present in the phospholipid, triacylglycerol and free fatty acid fractions of each serum. Other fatty acids (for example 20:0, 22:0, 22:4n-6 and 22:5n-3) were not detectable in either the sera or the cellular lipids. The phospholipids, triacylglycerols and free fatty acids were found to account for 78% of the total fatty acids present in the sera. Horse serum contained an approximately sixfold higher concentration of total fatty acids as compared to FCS. However, FCS contained slightly higher concentrations of *cis*-vaccenic acid,

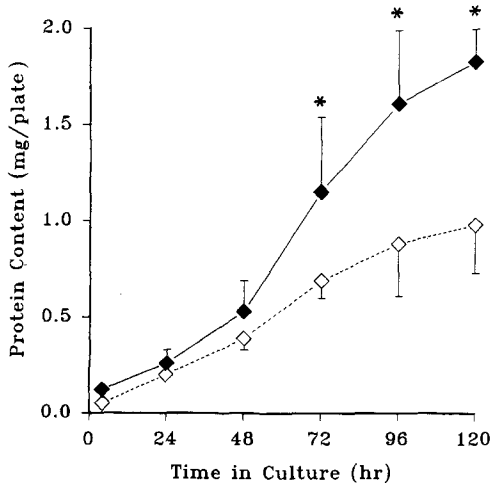

FIG. 1. Growth curves for HT29/219 cells grown in medium supplemented with fetal calf (◆) or horse (◇) serum (FCS or HS). Results shown represent the mean and 95% confidence limits of five replicates (FCS data) or three replicates (24–96 h HS data), or half the range for two replicates (4 h and 120 h HS data). In some cases the 95% confidence limits lie within the area covered by the symbols. *, Significant difference between DFC10- and DH10-grown cells, $P < 0.05$ (Mann-Whitney U-test).

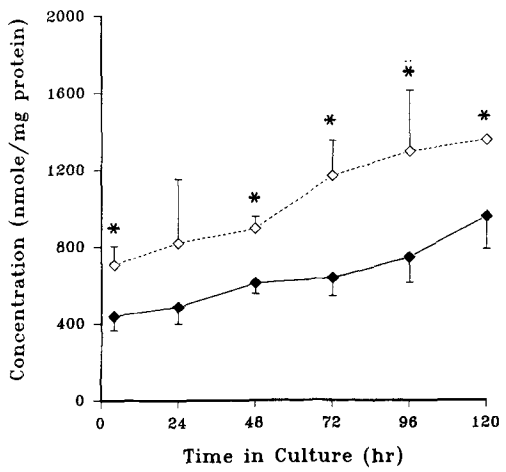

FIG. 2. Total fatty acid concentration in the phospholipid fraction of HT29/219 cells grown in medium supplemented with fetal calf (◆) or horse (◇) serum (FCS or HS). Results shown represent the mean and 95% confidence limits of five replicates (FCS data) or three replicates (24–96 h HS data), or half the range for two replicates (4 h and 120 h HS data). In some cases the 95% confidence limits lie within the area covered by the symbols. *, Significant difference between DFC10- and DH10-grown cells, $P < 0.05$ (Mann-Whitney U-test).

TABLE 1

Fatty Acid Concentrations of the Phospholipid, Triacylglycerol and Free Fatty Acid Fractions of Fetal Calf Serum and Horse Serum[a]

Fatty acids	Fatty acid concentration (nmol/mL)					
	PL		TG		FFA	
	FCS	HS	FCS	HS	FCS	HS
14:0	0.8	1.2	2.8	7.5	1.7	6.6
16:0	92.8	281.8	14.7	80.5	22.8	73.1
18:0	110.8	567.3	0.0	0.2	17.7	10.4
16:1	3.0	8.4	1.5	10.2	1.9	20.1
18:1 *c9*	80.2	182.6	6.7	64.7	18.2	89.6
18:1 *c11*	22.9	16.5	1.1	4.4	5.0	4.7
18:2	8.1	856.8	0.9	3.6	3.6	52.7
18:3	0.0	46.7	0.0	53.8	0.0	117.1
20:3	20.7	13.2	0.0	0.0	2.2	0.1
20:4	26.1	24.2	0.0	0.0	6.4	1.2
20:5	3.6	10.7	0.2	0.0	1.0	0.3
22:6	28.1	3.5	0.1	0.2	2.6	0.1

[a]PL, phospholipid; TG, triacylglycerol; FFA, free fatty acid; FCS, fetal calf serum; HS, horse serum. 18:1 *c9*, Oleic acid; 18:1 *c11*, *cis*-vaccenic acid; 18:2, linoleic acid; 18:3, α-linolenic acid; 20:3, 8,11,14-eicosatrienoic acid; 20:4, arachidonic acid; 20:5, 5,8,11,14,17-eicosapentaenoic acid; 22:6, docosahexaenoic acid. Results are the mean of two replicates.

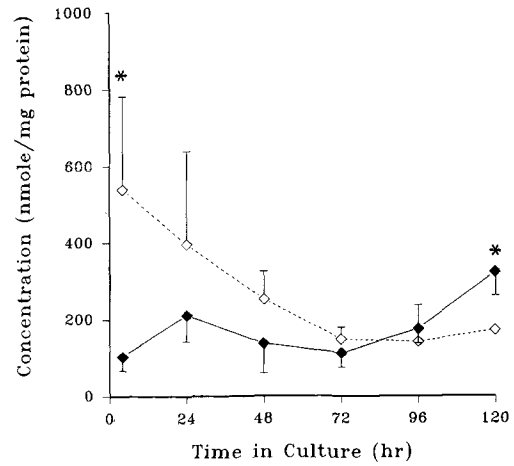

FIG. 3. Total fatty acid concentration in the triacylglycerol fraction of HT29/219 cells grown in medium supplemented with fetal calf (◆) or horse (◇) serum (FCS or HS). Results shown represent the mean and 95% confidence limits of five replicates (FCS data) or three replicates (24–96 h HS data), or half the range for two replicates (4 h and 120 h HS data). In some cases the 95% confidence limits lie within the area covered by the symbols. *, Significant difference between DFC10- and DH10-grown cells, $P < 0.05$ (Mann-Whitney U-test).

and the long-chain polyunsaturated acids 20:4n-6, 20:3n-6 and 22:6n-3. HS, on the other hand, contained considerably more of the essential fatty acids linoleic and α-linolenic, which were present in FCS at very low concentrations.

The sum of total fatty acids present in the cellular phospholipid fraction during growth in culture is shown in Figure 2. The corresponding data for the triacylglycerol fatty acids is shown in Figure 3. It was found that the fatty acid content of cellular phospholipids rose gradually with time in culture for both DFC10- and DH10-grown cells. The concentration of fatty acids in the triacylglycerol fraction of cells grown in DH10 was high at 4–24 h, after which time the levels dropped. In cells grown in DFC10, the fatty acid concentration of triacylglycerols rose in the first 24 h of growth, but then declined between 48 and 72 h.

Tables 2 and 3 show the fatty acid profiles of cellular phospholipids and triacylglycerols throughout growth in DFC10 and DH10, respectively. Levels of polyunsaturated

TABLE 2

Fatty Acid Composition of Phospholipids and Triacylglycerols in the HT29/219 Cells During Growth in Medium Supplemented with 10% Fetal Calf Serum[a]

Fatty acid	Time in culture (h)					
	4	24	48	72	96	120
			Phospholipids (%)			
14:0	2.0 ± 0.1	1.9 ± 0.3	2.4 ± 0.8	2.7 ± 0.3	2.4 ± 0.3	2.1 ± 0.2
16:0	15.6 ± 0.8	14.0 ± 4.9	17.9 ± 4.0	17.7 ± 3.6	16.6 ± 2.9	15.6 ± 2.2
18:0	8.1 ± 1.3	12.4 ± 1.2	13.1 ± 2.5	10.2 ± 2.8	9.0 ± 3.0	8.7 ± 3.0
Total SFA	25.7 ± 0.8	28.3 ± 3.5	33.3 ± 7.3	30.6 ± 6.7	28.0 ± 5.9	26.4 ± 5.0
16:1	12.8 ± 0.5	6.7 ± 4.7	10.5 ± 0.8	15.0 ± 0.7	21.0 ± 3.8	22.7 ± 3.0
18:1 *c9*	33.8 ± 2.9	26.7 ± 3.6	30.4 ± 2.3	36.6 ± 3.7	37.4 ± 2.8	37.9 ± 1.7
18:1 *c11*	3.2 ± 0.8	4.2 ± 1.2	3.6 ± 0.1	3.5 ± 0.3	3.5 ± 0.3	3.8 ± 0.3
Total MUFA	49.8 ± 3.7	37.6 ± 1.7	44.5 ± 3.2	54.8 ± 4.1	61.9 ± 6.8	64.4 ± 5.7
18:2	1.4 ± 2.5	2.8 ± 1.6	2.9 ± 0.7	1.4 ± 0.6	0.8 ± 0.9	0.8 ± 0.7
18:3	0	0	0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:3	3.0 ± 0.2	3.8 ± 0.5	2.5 ± 0.5	1.9 ± 0.5	1.4 ± 0.2	1.3 ± 0.2
20:4	12.4 ± 1.6	16.5 ± 5.1	9.0 ± 2.7	5.6 ± 1.6	3.8 ± 0.4	3.5 ± 0.6
20:5	3.9 ± 0.6	6.6 ± 3.6	4.1 ± 0.7	2.6 ± 0.6	1.6 ± 0.2	1.5 ± 0.3
22:6	3.8 ± 1.9	4.5 ± 1.6	3.7 ± 0.3	3.1 ± 0.5	2.4 ± 0.5	2.0 ± 3.0
Total PUFA	24.5 ± 3.2	34.1 ± 3.0	22.1 ± 4.2	14.6 ± 2.6	10.1 ± 1.3	9.2 ± 1.6
			Triacylglycerols (%)			
14:0	5.7 ± 3.4	3.5 ± 0.5	3.9 ± 0.7	4.2 ± 0.3	4.7 ± 0.9	4.5 ± 0.4
16:0	25.6 ± 5.0	21.3 ± 3.0	21.9 ± 0.7	24.6 ± 5.1	22.4 ± 4.2	20.2 ± 3.0
18:0	4.5 ± 2.6	11.7 ± 1.5	15.6 ± 3.4	12.8 ± 4.6	8.1 ± 6.7	7.0 ± 4.5
Total SFA	35.7 ± 6.3	36.4 ± 3.8	41.4 ± 4.5	41.5 ± 9.7	35.2 ± 10.1	31.6 ± 7.4
16:1	18.9 ± 7.3	9.0 ± 2.7	9.6 ± 1.5	12.2 ± 4.6	19.6 ± 7.7	21.4 ± 8.5
18:1 *c9*	36.8 ± 10.7	38.7 ± 3.2	39.0 ± 5.4	41.8 ± 6.0	41.3 ± 3.2	42.4 ± 2.1
18:1 *c11*	2.3 ± 3.2	4.1 ± 1.3	2.5 ± 2.0	1.6 ± 1.8	2.8 ± 0.5	3.4 ± 0.7
Total MUFA	58.0 ± 13.0	51.8 ± 1.8	51.0 ± 5.6	55.7 ± 9.0	63.7 ± 10.4	67.1 ± 8.4
18:2	1.2 ± 5.2	1.5 ± 1.6	0.7 ± 0.7	0.2 ± 0.3	0.1 ± 0.2	0.4 ± 0.7
18:3	0	0	0	0	0	0.1 ± 0.1
20:3	1.4 ± 1.4	2.3 ± 0.5	1.3 ± 0.5	0.4 ± 0.5	0.2 ± 0.1	0.2 ± 0.1
20:4	1.0 ± 2.5	2.0 ± 0.5	1.0 ± 0.5	0.4 ± 0.3	0.1 ± 0.2	0.1 ± 0.1
20:5	0.2 ± 0.6	0.7 ± 0.6	0.2 ± 0.2	0	0	0
22:6	2.6 ± 3.4	5.2 ± 3.1	4.3 ± 2.8	1.7 ± 0.7	0.7 ± 0.2	0.5 ± 0.3
Total PUFA	6.3 ± 7.1	11.8 ± 5.3	7.5 ± 3.5	2.8 ± 1.1	1.1 ± 0.4	1.3 ± 1.0

[a]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. See legend in Table 1 for shorthand designations of fatty acids. Results shown represent the mean (mol%) and 95% confidence limits of five replicates.

acids were increased in the lipids of DH10-grown cells as compared to DFC10-grown cells. This increase was mainly due to significantly higher levels of linoleic and α-linolenic acids being incorporated into lipids of DH10-grown cells. These two acids accounted for approximately 40% of total fatty acids in the phospholipids and 20% of total fatty acids in the triacylglycerols of DH10-grown cells throughout culture, whereas they were either absent (or present at low levels) in DFC10-grown cells (Tables 2 and 3). The latter, however, contained increased amounts of C_{20} acids as well as 22:6n-3, as compared to DH10 grown cells. Levels of these acids were at their maximum 24 h after growth and decreased afterwards. In cells grown with either serum supplement, the proportion of monounsaturated acids increased during culture (Tables 2 and 3).

For both media analyzed, the concentration of all free fatty acids fell to <10% of their initial values over 24–48 h. This was confirmed using ^{14}C-labeled 18:1n-9, 18:2n-6 and 20:4n-6 added to DFC10 medium at the start of the experiment. After 48 h in culture, only 10% of the original radioactivity was detected in the medium. Within the cells, 90% of the radioactivity taken up from the medium was found to be associated with the phospholipids and triacylglycerols, the former containing most of the label. The results are presented here as the specific activity of label in each lipid fraction (*i.e.*, the amount of label incorporated per unit mass of total fatty acids in each lipid fraction) (Fig. 4). This gives an indication of incorporation relative to cellular concentration of the different lipids. No difference was observed in the incorporation of [1-^{14}C]linoleic acid into the phospholipids and triacylglycerols (Fig. 4b). Incorporation of label into both lipid classes was most rapid in the first 24 h, reaching a maximum at 24–48 h and falling thereafter. When the HT29/219 cells were incubated with [1-^{14}C]arachidonic acid, the amount of label incorporated per unit mass of lipid was lower for the triacylglycerol fraction as compared to the phospholipid fraction (Fig. 4a). However, more [1-^{14}C]oleic acid was incorporated into the triacylglycerol fraction compared to the phospholipids (Fig. 4c). When these experiments were repeated at a later time, the same trend was observed for the incorporation of the acids, confirming the results reported here.

TABLE 3

Fatty Acid Composition of Phospholipids and Triacylglycerols in the HT29/219 Cells During Growth in Medium Supplemented with 10% Horse Serum[a]

Fatty acid	Time in culture (h)					
	4	24	48	72	96	120
			Phospholipids (%)			
14:0	3.1 ± 0.1	1.4 ± 0.5	2.0 ± 0.4	3.0 ± 1.4	3.3 ± 2.3	3.2 ± 0.6
16:0	18.6 ± 0.9	14.9 ± 2.1	12.2 ± 2.7	13.5 ± 3.0	12.3 ± 4.6	9.8 ± 0.9
18:0	17.1 ± 2.4	15.7 ± 0.8	14.9 ± 1.1	11.6 ± 0.8	10.0 ± 1.1	8.9 ± 0.8
Total SFA	38.8 ± 3.4	32.0 ± 3.4	29.1 ± 4.2	28.2 ± 3.5	25.5 ± 5.7	22.0 ± 0.6
16:1	2.8 ± 1.3	3.1 ± 0.5	3.7 ± 2.6	5.9 ± 3.2	9.9 ± 4.2	14.7 ± 1.7
18:1 *c9*	16.4 ± 0.8	17.5 ± 1.7	21.3 ± 1.8	24.3 ± 1.4	26.5 ± 1.4	25.9 ± 0.8
18:1 *c11*	1.5 ± 0.4	1.8 ± 0.1	1.8 ± 0.2	1.3 ± 1.5	2.2 ± 0.4	2.8 ± 0.2
Total MUFA	20.7 ± 2.5	22.4 ± 1.2	26.9 ± 2.3	31.5 ± 4.1	38.5 ± 5.7	43.3 ± 0.8
18:2	31.0 ± 0.4	33.0 ± 1.5	31.7 ± 1.3	31.2 ± 0.8	28.8 ± 0.5	29.0 ± 1.1
18:3	5.1 ± 0.4	8.2 ± 1.3	8.9 ± 1.7	7.1 ± 0.3	5.4 ± 0.2	3.9 ± 0.1
20:3	0.8 ± 0.3	0.7 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.3 ± 0.4	0.4 ± 0.5
20:4	2.1 ± 0.1	2.1 ± 0.3	1.6 ± 0.2	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 1.1
20:5	1.1 ± 0.2	1.2 ± 0.1	0.9 ± 0.1	0.5 ± 0.4	0.4 ± 0.2	0.3 ± 0.1
22:6	0.4 ± 0.1	0.4 ± 0.2	0.2 ± 0.0	0.2 ± 0.4	0	0.1 ± 0.2
Total PUFA	40.5 ± 0.9	45.6 ± 2.1	44.0 ± 3.4	40.4 ± 1.8	35.9 ± 0.6	34.7 ± 1.5
			Triacylglycerols (%)			
14:0	4.9 ± 0.9	2.8 ± 0.6	2.7 ± 1.2	3.7 ± 1.4	5.5 ± 4.3	5.8 ± 1.1
16:0	27.2 ± 0.1	21.6 ± 6.5	16.8 ± 3.1	15.9 ± 4.9	14.4 ± 6.6	9.4 ± 1.0
18:0	11.7 ± 1.1	11.9 ± 3.6	12.1 ± 0.9	10.7 ± 1.1	5.7 ± 1.7	2.9 ± 0.4
Total SFA	43.8 ± 0.1	36.2 ± 9.3	31.6 ± 5.2	30.3 ± 6.6	25.7 ± 9.9	18.2 ± 1.6
16:1	3.0 ± 1.4	4.0 ± 1.5	4.4 ± 1.0	5.8 ± 0.9	12.1 ± 3.8	19.4 ± 2.2
18:1 *c9*	31.6 ± 4.8	32.7 ± 6.3	35.0 ± 3.7	37.2 ± 4.9	41.3 ± 4.7	39.9 ± 1.7
18:1 *c11*	2.2 ± 0.1	1.7 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.8 ± 0.3	2.4 ± 0.2
Total MUFA	36.8 ± 3.5	38.5 ± 4.5	40.8 ± 2.9	44.5 ± 4.2	55.2 ± 2.1	61.7 ± 0.3
18:2	13.8 ± 0.4	15.6 ± 4.1	17.1 ± 1.9	16.0 ± 3.0	12.7 ± 5.1	14.0 ± 0.6
18:3	4.2 ± 2.4	9.4 ± 7.0	10.1 ± 2.1	8.8 ± 1.1	6.4 ± 4.5	6.0 ± 1.2
20:3	1.0 ± 0.9	0.1 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	0	0
20:4	0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0	0
20:5	0.1 ± 0.1	0	0	0	0	0
22:6	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.4	0.2 ± 0.2
Total PUFA	19.4 ± 3.5	25.3 ± 10.8	27.6 ± 4.0	25.2 ± 3.6	19.2 ± 8.9	20.1 ± 2.0

[a]See Table 2 for abbreviations. Results shown represent the mean (mol%) and 95% confidence limits of three replicates.

DISCUSSION

The fatty acid compositions determined for total extracted lipids from the two sera closely resemble those reported by other investigators (1,16–19). However, some differences were observed for a number of acids. The percentage composition of stearic acid found in FCS in this study was twice that found by other investigators, whereas the percentage composition of palmitoleic acid was 2–3-fold lower. The percentage composition of α-linolenic acid in HS varied from 1.5% in one study (1) to 10.2% in the present work. These discrepancies may be due to differences in fatty acid concentrations of different batches of serum as observed by Stoll and Spector (16). However, no significant differences were found in this study for the different batches of sera used.

The gradual rise in the fatty acid content of the phospholipid fraction of both DFC10- and DH10-grown cells with time in culture indicates a higher rate of phospholipid synthesis relative to protein synthesis within the cells or, alternatively, a higher degee of protein catabolism. Measurement of cellular DNA may provide useful information regarding the most likely explanation. In contrast, the level of total acids in the triacylglycerol fraction of the cells followed a different pattern which depended on the serum supplement used. The results indicate a greater initial incorporation of fatty acyl moieties into the cellular triacylglycerol fraction when cells were grown with HS. This is probably due to stimulation of triacylglycerol synthesis by higher concentrations of fatty acids in this serum (20). We have not considered the role of the free fatty acids within the cells, because levels of this lipid class were very low (<5% of total cellular fatty acids for DFC10-grown cells and <10% for DH10-grown cells) in every experiment, suggesting a high esterification rate.

The phospholipid fraction of cells grown with FCS was particularly high in monounsaturated acids and low in polyunsaturated ones, whereas the reverse was true in cells grown with HS. These alterations in the fatty acyl composition of cellular phospholipids as a result of changing the serum supplement confirm the reports on MDCK and 3T3 cells (16). Such alterations are expected to influence the cell membrane. For example, Simon *et al.* (21) have shown that increased unsaturation of the phospho-

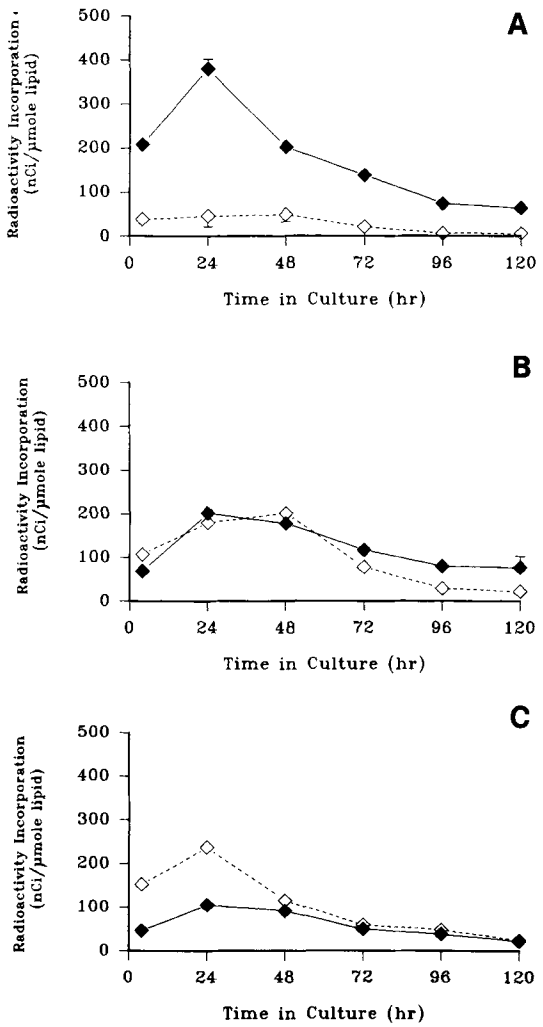

FIG. 4. Specific activity of incorporated (a) [1-^{14}C]arachidonic acid; (b), [1-^{14}C]linoleic acid; and (c) [1-^{14}C]oleic acid in the phospholipids (◆) and triacylglycerols (◇) of HT29/219 cells during growth in medium supplemented with 10% fetal calf serum and 15 nCi/mL of radiolabeled fatty acid. Results shown in (a) and (b) represent the mean and half the range of duplicate calculations (in most cases the range lies within the area covered by the symbols). The results shown (c) were calculated from a single measurement. Each point was derived by dividing radioactivity measurements (nCi/mg protein) by the means of cellular total phospholipid or triacylglycerol concentrations (nmoles/mg protein) shown in Figures 2 and 3.

lipid fatty acyl chains leads to an increase in the fluidity of the membrane. The role of membrane fluidity in relation to cell membrane functions such as carrier-mediated transport, activity of membrane-bound enzymes, binding properties of cellular receptors and phagocytosis has been well-documented (22). As a result, it may not be acceptable to compare biochemical studies carried out on cells grown in different serum-supplemented media. This is particularly important with a view to recent attempts to encourage the use of "cheaper" horse and adult bovine serum supplements instead of the most common, but costly FCS (23). Furthermore, care must be taken when comparing *in vitro* and *in vivo* systems with regard to lipid metabolism. An illustration of this can be found in the findings of Isseroff *et al.* (19) and Madison *et al.* (24). Both groups showed that murine keratinocytes grown in culture supplemented with FCS have much less linoleic acid than uncultured fresh cells due to the lower levels of this fatty acid in FCS as compared to mouse serum. They suggest that this finding may be partly responsible for the incomplete differentiation pattern of these cells *in vitro*.

Radioactivity taken up from the medium was found to be associated with the cellular phospholipids and triacylglycerols, the former containing most of the label. Results showing the specific activity of the label in each lipid fraction suggest some selectivity in the incorporation of [1-^{14}C]oleic acid into the triacylglycerols, no specificity for incorporation of [1-^{14}C]linoleic acid into the two lipid classes, and a higher selectivity for incorporation of [1-^{14}C]arachidonic acid into the phospholipid fraction. As the same amount of label was removed from the medium when cells were incubated with any of the labeled acids, there does not seem to be any selectivity in the uptake of the different acids. Rather, selectivity is more likely to be involved in (i) their transport within the cells; (ii) their activation by coenzyme A; or (iii) their final incorporation into the different lipids. As the total amount of label detected in the cellular lipids was very similar for the three acids, (i) and (ii) seem less likely to be appropriate in these cells. Therefore, the most probable point of selectivity appears to be in the activity of the acyltransferase enzymes involved in glycerolipid synthesis. Our results have shown that more polyunsaturated acids are channelled into phospholipid production and less into triacylglycerol synthesis.

Athough the phospholipid fatty acyl composition of the cells reflected the free fatty acid composition of the added serum, some major differences were observed (Tables 1, 2 and 3). The fatty acids found to be less affected by the medium free fatty acid composition were stearic acid, linoleic acid and α-linolenic acid. It was found that the cells accumulated linoleic acid so that the medium contained lower proportions of this as the free acid as compared to the cells. Conversely, the cells incorporated less α-linolenic acid than the quantity present in the medium free fatty acid fraction. A similar finding of lower 18:3n-3 incorporation compared to 18:2n-6 has been reported in skin fibroblasts and in cells of neuronal origin (1). Clouet *et al.* (25) have reported that α-linolenic acid is oxidized by rat liver mitochondria at a greater rate than linoleic acid. One pathway of 18:3n-3 metabolism is its repeated desaturation and elongation to yield 20:4n-3 and 22:5n-3. Although our results showed some evidence for this conversion (see below), the rate of this conversion did not exceed that of 20:4n-6 synthesis from 18:2n-6.

The differences observed in composition of the abovementioned fatty acids may be due to different rates of incorporation and/or mobilization of these acids. However, when we consider the fatty acid composition of total lipids in the sera, it is observed that the cellular profiles reflect this more closely than the free fatty acid composition of sera. This is indicative of uptake of lipids other than free fatty acids by the cells and is discussed in more detail below.

In cells grown with either serum supplement, the acylation of all C_{20} polyunsaturated fatty acids and 22:6n-3 into cellular phospholipids and triacylglycerols increased in the first 24 h, probably as a result of rapid uptake from the medium. The majority of the polyunsaturated fatty

acids were incorporated into the phospholipids. After 24 h these acids were mobilized from cellular lipids, possibly for utilization in other cellular functions, such as production of prostaglandins and leukotrienes. Due to the important function of these acids in cellular metabolism, this finding is an important factor to consider for timing of biochemical studies in cultured cells.

Linoleic acid was progressively acylated into the phospholipids for up to 48–72 h, after which time levels remained relatively constant. It would therefore seem that the cells were continuously taking up more of this acid from the medium throughout the culture period. Analysis of medium free fatty acids showed that practically all of the free linoleic acid was removed from the medium by 24–48 h. Our results thus indicate that linoleic acid from other lipid sources in the medium was taken up by the cells. These other sources are triacylglycerols, phospholipids and cholesteryl esters, mostly contained in lipoproteins. Evidence for this is provided by the quantitative analysis of fatty acids in medium and cellular lipids. The concentration of 18:2n-6 in the free fatty acid fraction of DH10 was 50 nmoles/plate at the start of the 120-h culture period. At 4 h the amount of this acid present as cellular phospholipid and triacylglycerol (no free fatty acid) was approximately 10 nmole/plate. This rose to 410 nmoles as the cells multiplied in culture. Therefore, the cells took up 400 nmole of linoleic acid during the 4–120 h culture period. Analysis of horse serum lipids revealed that there was approximately 850 nmole/plate of linoleic acid available in the phospholipid fraction of the medium at the start of the culture in addition to the 50 nmole already mentioned as the free acid (Table 1). In other words, 80% of the total linoleic acid present in HS was in the phospholipids and only 5% was present as the free acid. Despite this abundant supply of linoleic acid in the phospholipid fraction of the medium, we were unable to detect any free linoleic acid in the medium after 48 h. Therefore, the cells must have been taking up either intact lipids, as previously reported for a number of mammalian cells (1), or if prior hydrolysis of these lipids did occur in the medium, as has been suggested for human diploid cells (3), then uptake of the released fatty acids was very rapid. Furthermore, labeled fatty acid experiments revealed that no significant efflux of the label into the medium occurred after the take up phase was complete. Further studies using radiolabeled phospholipids and triacylglycerols are necessary to confirm these findings.

When similar comparisons of cellular and medium fatty acid concentrations are carried out for other fatty acids, it is found that for a number of acids, even when total fatty acids in the medium are taken into consideration, there is not sufficient levels in the medium to account for the levels accumulated in the cells during the culture period. This indicates that these acids are being actively synthesized within the cells. The calculated data for all fatty acids analyzed are presented in Table 4. In our analysis of the data, a higher concentration of fatty acids incorporated into the cells compared to that available in the medium indicates synthesis. Therefore, a positive value in Table 4 indicates that there is enough fatty acids in the medium to account for all the phospholipid and triacylglycerol fatty acids measured in the cells. Similarly, a negative value indicates that there is not enough fatty acid in the medium to account for all cellular fatty acids, and therefore the cell is actively synthesizing fatty acids.

Table 4 provides evidence that cells grown with FCS synthesize almost all the fatty acids by the end of the culture period. There is substantial synthesis of the monounsaturated acids as early as 48 h after plating, thus indicating a high Δ9 desaturase activity. The only acids for which no evidence of cellular biosynthesis was found were, as expected, the essential acids 18:2n-6 and 18:3n-3, as well as 20:3n-6. The synthesis of the polyunsaturated acids, 20:4n-6, 20:5n-3 and 22:6n-3, indicates the presence, although minimal, of Δ5 and Δ4 desaturase, as well as elongase enzymes in the HT29/219 cells. Although elongases have been found in most cells in culture (1), the desaturases have been shown to be absent from a number of cell lines (26,27), the Δ4 desaturase having been found in only a few cases (1).

When cells were grown with HS, the only acids for which synthesis was established were palmitoleic, myristic and, to a lesser extent, *cis*-vaccenic acids (Table 4). Little, if any, of the large amount of cellular linoleic and α-linolenic acids were converted to the long-chain polyunsaturated acids analyzed. This is probably due to a low activity of the Δ6, Δ5 and Δ4 desaturase enzymes, plus the fact that a large load of linoleic and α-linolenic acids taken up from the medium compete with relatively small amounts of longer chain polyunsaturated acids that may be formed for incorporation into cellular lipids. Similar results were reported by Spector's group (1) studying human 1603 skin fibroblasts grown in medium supplemented with linoleic acid.

Although due to variation in extraction procedures, the absolute magnitude of the values presented in Table 4 may not be significant, the trends should be considered as important. The positive or negative sign of the values are also significant in that they indicate whether or not cellular fatty acid biosynthesis may have taken place during culture. However, it must be noted that although a negative value indicates synthesis, a positive sign does not exclude it. Although the results of the mass balance studies need to be verified by pulse-chase experiments using radioactive precursors such as [1-^{14}C]glucose or [1-^{14}C]acetate, they cannot be rationalized by any process other than *de novo* fatty acid synthesis. The concentration of fatty acids in the serum used to supplement the growth medium influenced the rate of biosynthesis and the time period after which synthesis was shown to occur.

Cellular protein measurements show that although the cells used in this study grow in medium supplemented with either fetal calf or horse sera, the type of serum used influences the rate of growth of the cells. A number of reports in the literature have shown a direct effect of some unsaturated acids on cellular growth (1,18,28,29). The present finding that cellular growth rates were similar for the first 48 h with either serum supplement suggests that there were sufficient fatty acids in both systems for growth and that the increased growth observed with FCS after 48 h was probably due to serum factors other than the lipid component. However, since 48 h is often the timepoint at which a medium change is performed in the routine culture of these cells, this particular observation may not be significant in routine work.

TABLE 4

Difference Between HT29/219 Cellular Fatty Acid Levels During Culture and the Levels Present in the Total Lipids of the Medium at the Start of the Experiment[a]

Fatty acid	Medium conc.[b]	Amount of acids in medium minus amount in cellular lipids				
		24 h	48 h	72 h	96 h	120 h
			Growth in DFC10			
14:0	7.9	5.4	−0.6	−15.6	−32.5	−54.2
16:0	134.0	115.6	71.9	−14.7	−116.5	−241.6
18:0	129.2	112.2	81.5	44.0	3.2	−54.0
16:1	10.6	6.2	−21.4	−106.6	−283.9	−511.2
18:1 *c9*	105.3	72.9	0.1	−191.6	−434.1	−788.1
18:1 *c11*	31.2	25.5	19.6	6.1	−16.8	−53.0
18:2	16.8	13.4	7.9	7.5	6.9	1.4
18:3	1.1	1.0	1.1	0.6	0.4	0.4
20:3	23.9	19.6	16.4	11.2	8.0	1.6
20:4	35.2	20.1	11.6	0.3	−4.9	−21.0
20:5	8.3	1.7	−3.0	−8.5	−9.3	−15.6
22:6	34.1	28.1	21.7	11.9	6.3	−1.4
			Growth in DH10			
14:0	28.1	25.9	20.8	2.1	−12.5	−22.2
16:0	546.1	519.1	501.1	434.8	404.0	413.8
18:0	697.7	672.3	643.5	603.2	586.2	583.5
16:1	52.0	45.2	36.2	0.3	−74.4	−174.0
18:1 *c9*	401.4	361.8	307.9	183.6	63.4	5.2
18:1 *c11*	31.9	28.7	25.3	21.2	5.5	−7.7
18:2	1070.4	1018.3	957.9	818.1	741.5	676.3
18:3	329.1	310.4	290.9	265.7	262.0	270.1
20:3	16.0	15.3	14.1	13.1	12.7	11.2
20:4	27.6	24.9	22.8	20.3	17.5	14.9
20:5	13.2	11.6	10.4	9.5	8.7	9.4
22:6	4.9	4.4	4.1	3.4	4.7	3.7

[a]Abbreviations as in Table 1. DFC10, Dulbecco's modification of Eagle's essential medium enriched with 10% fetal calf serum; DH10, Dulbecco's modification of Eagle's essential medium enriched with 10% horse serum. Negative values indicate cellular biosynthesis.

[b]Concentration of total fatty acids in the medium at the start of the experiment (nmole/plate). It should be noted that although in subsequent fractionation steps, potential lipid sources other than phospholipids and triacylglycerols (*e.g*, cholesteryl esters, mono- and diacylglycerols) were not measured, when total fatty acid measurements were made for the sera the amounts of these lipids were also included.

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Effects of Aging on the Content, Composition and Synthesis of Sphingomyelin in the Central Nervous System

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Sphingomyelin (SPH) content and composition in different regions of the brain were analyzed in 2.5, 21.5 and 26.5-month-old rats. SPH content increased in the cerebral hemispheres, cerebellum and medulla oblongata plus pons as age increased. The highest SPH content was observed in 26.5-month-old rats, with values increasing by 1.74, 2.75 and 0.88-fold, respectively, over 2.5-month-old rats. The SPH fatty acid composition of brains from aged rats was markedly different from that of adult rats. Between 2.5 and 26.5 months of age the monoenoic/saturated fatty acid ratio increased from 0.22, 0.30 and 0.54 to 0.54, 0.68 and 1.03 in cerebral hemispheres, cerebellum and medulla oblongata plus pons, respectively. The percentage and content of fatty acids longer than 22 carbon atoms esterified to SPH increased with age from 18, 26 and 44 to 48, 52 and 62 mole % in cerebral hemispheres, cerebellum and medulla oblongata plus pons in 26.5-month-old rats. In subcortical white matter from aged rats, monoenoic 22–26 carbon atom fatty acids increased more than the saturated ones in 21.5-month-old rats relative to 2.5-month-old rats. *In vitro* synthesis of SPH from [^{3}H]choline and [^{3}H]palmitic acid in cerebral cortex and cerebellum showed no significant differences between adult rats and those 21.5 months of age. In cerebellum and in cerebral cortex, [^{14}C] serine incorporation increased in aged rats. The results suggest that aging induces increases in both SPH content and in the monoenoic/saturated fatty acid ratio. These increases are quantitatively different in all brain regions analyzed.
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The molecular basis of aging in nervous tissue is still largely unknown. Cell membrane composition alters during the aging process with subsequent alterations in membrane biophysical properties. Polyunsaturated acyl groups of glycerophospholipids have been shown to decrease in the brain and retina of rodents (1–3). Many membrane-bound enzymes and transport systems are affected by alterations in the properties of the lipid bilayer (4,5). The synthesis of sphyingomyelin (SPH), one of the phospholipids of mammalian cell membranes (6–8), occurs by the transfer of the phosphocholine group from phosphatidylcholine to ceramide (9). Inserted into biological membranes, sphingomyelin generally increases membrane viscosity (9). SPH is also thought to have a high affinity for cholesterol (10–12). In rodents, aging is associated with a progressive increase in brain weight, cholesterol content, cholesterol to phospholipid molar ratio and myelin content (13).

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Abbreviations: CC, cerebral cortex; CNS, central nervous system; FA, fatty acids; GLC, gas-liquid chromatography; M/S, monoenoic-to-saturated FA ratio; MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; SFA, saturated fatty acids; SPH, sphingomyelin; SWM, subcortical white matter; TLC, thin-layer chromatography; TPL, total phospholipid; UV, ultraviolet.

In the present work the effect of aging on sphingomyelin content and composition in different regions of the brain was examined. The synthesis of this sphingolipid from [^{3}H]palmitic acid, [^{3}H]choline and [^{14}C]serine also was studied. The level of SPH in the brain increased with age, and the fatty acid composition of SPH was dramatically affected by age.

MATERIALS AND METHODS

[^{14}C(U)]L-Serine (specific activity, 168 mCi/mmol), [*methyl*-^{3}H]choline chloride (specific activity, 69.5 Ci/mmol), palmitic acid [9,10-^{3}H(N)] (specific activity, 15.2 Ci/mmol) and Omnifluor were purchased from New England Nuclear (Boston, MA). All other reagents were of analytical grade. Wistar-strain rats were kept under controlled environmental conditions and were fed a standard pellet diet. The cerebral hemispheres, cerebellum and medulla oblongata plus pons were obtained from a group of rats 2–3 (2.5) or 26–27 (26.5) months old. From a second group of rats, aged 2–3 (2.5) or 21–22 (21.5) months, the cerebral cortex and subcortical white matter were removed in addition to the above-mentioned regions. Rats were killed by decapitation.

For metabolic studies, the cerebral cortex and cerebellum of 3 and 21.5-month-old rats were used. The tissues were minced to obtain about 1 mm^3 fragments and transferred to conical tubes containing minimum essential medium (Gibco Lab, Grand Island, NY) supplemented with 2.2 g/L of $NaHCO_3$, 5% fetal calf serum (Sigma Chemical Co., St. Louis, MO) and L-glutamine, at a final concentration of 2 mM (10 mL of medium per cortex or cerebellum). The tissue fragments were aspirated and expelled 10 times using a 1-mm diameter Pasteur pipette according to Alperin *et al.* (14). After sedimentation, particles were resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4). Samples (about 100 μg of protein/sample) were incubated for 15, 30 and 90 min at 37°C in the same medium using 5 μCi of [^{14}C]serine or [^{3}H]choline or 20 μCi of [^{3}H]palmitic acid in a final volume of 0.2 mL.

In all cases lipids were extracted from tissue as described by Folch *et al.* (15). Unlabeled DL-serine (1%) or choline (1%) (or choline [1%]) were added to the upper phases to wash lipid extracts labeled with [^{14}C]serine or [^{3}H] choline, respectively. Phospholipids were isolated by two-dimensional thin-layer chromatography (TLC) (16) and located by exposure to iodine vapors. The fractions were scraped off and transferred to tubes for measurement of lipid phosphorus (16) or to vials containing 0.4 mL water and 10 mL of 0.5% Omnifluor in toluene/triton X-100 (4:1, vol/vol) to measure radioactivity by liquid scintillation counting.

Sphingomyelin was isolated by two-dimensional TLC to determine the fatty acid composition. The plates were sprayed with 2′7′-dichlorofluorescein, and the spots were located under ultraviolet (UV) light. Methyl esters were prepared (17) and analyzed by gas-liquid chromatography (GLC). Two glass columns (2 m × 2 mm i.d.) packed with

TABLE 1

Total Phospholipid and Sphingomyelin Content of Cerebral Hemispheres, Cerebellum and Medulla Oblongata Plus Pons from Adult and Aged Rats[a]

	nmol/mg protein		
	Sphingomyelin (SPH)	Total phospholipid (TPL)	TPL/SPH[b]
Cerebral hemispheres			
2.5 mo (5)[c]	23.61 ± 7.52 (4.6%)[d]	572.91 ± 48.81	24.3 ± 3.6
21.5 mo (4)	23.44 ± 1.16 (4.2%)	551.92 ± 68.10	23.5 ± 1.4
26.5 mo (3)	64.62 ± 2.48 (11.4%)	568.62 ± 53.54	8.8 ± 0.5[e]
Cerebellum			
2.5 mo (5)	24.86 ± 4.31 (5.0%)	517.12 ± 19.91	20.8 ± 1.7
21.5 mo (4)	27.81 ± 5.43 (5.9%)	524.61 ± 92.70	18.9 ± 2.5
26.5 mo (3)	93.32 ± 6.05[e] (17.4%)	537.00 ± 11.20	5.8 ± 0.2[e]
Medulla oblongata + pons			
2.5 mo (5)	37.53 ± 14.0 (5.2%)	728.83 ± 22.41	19.4 ± 3.2
21.5 mo (4)	45.66 ± 13.2 (6.4%)	713.08 ± 54.41	15.6 ± 2.3
26.5 mo (3)	70.51 ± 8.3[f] (10.2%)	694.40 ± 6.44	9.9 ± 0.7[e]

[a]The amounts of SPH and TPL [nmol/mg protein] are presented as mean values ± SD.
[b]SD from TPL/SPH ratio were calculated according to Johnson and Kotz (19).
[c]Numbers in parentheses refer to the numbers of samples. Each sample corresponding to 21.5 or 26.5-month-old rats consisted of two or three cerebrum, cerebellum and medulla oblongata plus pons, respectively.
[d]The numbers in parentheses refer to the percentage composition of SPH with respect to total phospholipid.
[e,f]Data are compared to adult rats using the Student's *t*-test ([e]$P < 0.001$; [f]$P < 0.025$).

15% OV-275 on 80–120 Chromosorb WAW (Varian, Sunnyvale, CA) were connected to two flame ionization detectors operated in the dual-differential mode. A linear (5°C/min) temperature program, starting at 160°C and ending at 220°C, was used. The oven temperature was held constant at 220°C for 20 min to permit the analysis of very long-chain fatty acids. The injector and detector temperatures were 220 and 230°C, respectively. Protein content was determined by the method of Lowry *et al.* (18). Statistical analysis was performed using Student's *t*-test, with the values representing the mean ± standard deviation (SD) of the total number of samples indicated in each legend. In the case of the ratios, the SD was calculated according to the *ad-hoc* statistical treatment of Johnson and Kotz (19):

$$SD = \bar{x}_1/\bar{x}_2 \times [(SD_1)^2/n_1 \times (\bar{x}_1)^2] + [(SD_2)^2/n_2 \times (\bar{x}_2)^2]$$

RESULTS AND DISCUSSION

Total phospholipid (TPL) and SPH contents were measured in different brain regions of adult (2.5 mo) and aged (21.5 and 26.5 mo) rats. TPL content in adult rats was higher in medulla oblongata plus pons than in cerebral hemispheres and cerebellum (Table 1). Sphingomyelin represented about 5% of the total phospholipid in the different brain regions studied. When compared to the SPH concentration in adult rats, the SPH concentration (expressed as the amount of lipid phosphorus per mg of tissue protein) in 26.5-month-old rats increased by a factor of 2.7, 3.7 and 1.9, respectively, in cerebral hemispheres, cerebellum and medulla oblongata plus pons. However, the SPH content increased only slightly in 21.5-month-old rats, showing that the most dramatic changes occur between 21.5 and 26.5 months of age. The percentage distribution of SPH with respect to total phospholipid content also increased with age reaching the highest value in medulla oblongata plus pons. The ratio of total phospholipid to sphingomyelin content diminished with age, from 24.3, 20.8 and 19.4 to 8.8, 5.8 and 9.9 in cerebral hemispheres, cerebellum and medulla oblongata plus pons, respectively. Total phospholipid content was not affected by aging, which is in agrement with results from previous studies (20,21).

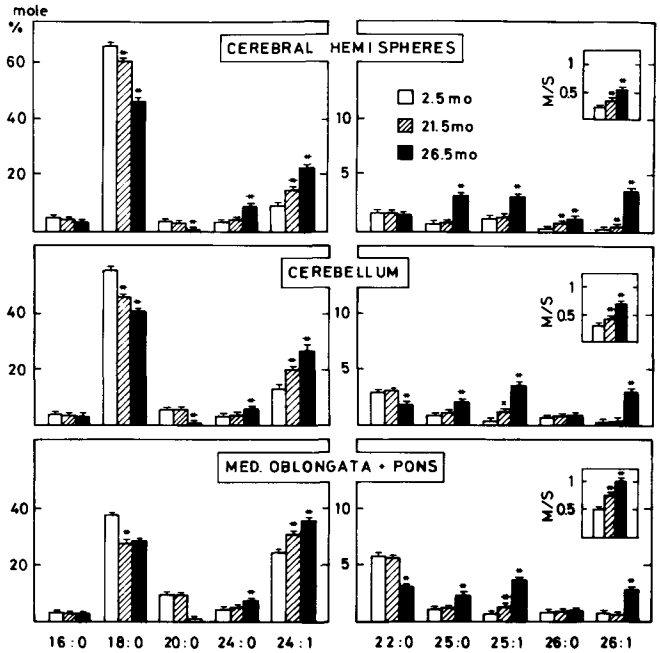

FIG. 1. Fatty acid composition of sphingomyelin in different regions of the central nervous system from adult and aged rats. Results are presented as means ± SD from a number of samples, as indicated in Table 1. Data are compared to controls by using the Student's *t*-test. +, $P < 0.025$; *, $P < 0.001$; M/S, monoenoic-to-saturated FA ratio.

Figure 1 shows the SPH acyl group composition in dif-

ferent regions of the central nervous system. SPH contains mainly saturated and monounsaturated fatty acids. Stearic acid (18:0) was the major saturated SPH fatty acid and accounted for 38 to 66% of the total in all regions analyzed. Other quantitatively important saturated fatty acids were palmitic (16:0), arachidic (20:0), lignoceric (24:0) and behenic (22:0) acids (Fig. 1). The major very long-chain unsaturated fatty acid was nervoneate (24:1), which was more concentrated in medulla oblongata plus pons. The degree of unsaturation followed the order: cerebral hemispheres < cerebellum < medulla oblongata plus pons (Fig. 1, inset).

Aging produced a pronounced change in the fatty acid composition of SPH. The mole percentage distribution of stearic acid decreased at 26.5 months of age to 69, 74 and 78% of the adult rat values for cerebral hemispheres, cerebellum and medulla oblongata plus pons, respectively. By comparison, nervoneate increased with age, and showed the highest change of all monounsaturated fatty acids in rats aged 26.5 months (155, 105 and 145% over the adult rat values in the same regions, respectively). The total percentage of saturated fatty acids diminished and that of monounsaturated fatty acid increased; thus aging affected the ratio of monoenoic to saturated acyl groups, which increased in brain regions of older rats in the following order: cerebral hemispheres < cerebellum < medulla oblongata plus pons (Fig. 1, inset).

The content and composition of SPH were analyzed in the cerebral cortex and subcortical white matter of 2.5- and 21.5-month-old rats to determine whether the changes observed in whole cerebral hemispheres were due to variations produced in either region. Figure 2 shows that the most significant changes were observed in subcortical white matter. The content of SPH in 21.5-month-old rats increased about 27% in this region; however, no changes were observed in cerebral cortex of rats from the same age group. Total phospholipid content was not affected by aging in any region. Figure 2 also shows that the fatty acid composition of these two regions in aged rats is different from that in adult rats. The acyl groups of SPH from subcortical white matter showed more changes in their fatty acid composition than did those of the cerebral cortex. In addition, the ratio of monoenoic to saturated fatty acids increased in 21.5-month-old rats with respect to adult animals, as was observed in rats aged 26.5 months (Fig. 1, inset).

These findings suggest either that a different turnover of the molecular species of this sphingolipid takes place and/or that Δ9 desaturase may be stimulated by aging. In agreement with our observations, earlier studies have found that the acyl groups of ethanolamineglycerophospholipid in myelin from the older mice, rhesus monkeys and humans have higher proportions of monoenes (mainly 18:1 and 20:1) than those found in adult rats (22–24).

In Table 2 the contents of both saturated and monoenoic fatty acids of SPH from adult and aged rats are presented. Fatty acids with carbon chain lengths of 22 or more carbon atoms also are shown. Monounsaturated fatty acids amounted to approximately 17–32% of the total fatty acids in adult rats and increased by 5.4, 5.04 and 2.76 times in cerebellum, cerebral hemispheres and medulla oblongata plus pons, respectively, at 26.5 months. Significant changes also were observed in medulla oblongata plus pons at 21.5 months of age. Greater changes were observed in SPH content of subcortical white matter than in that of the cerebral cortex at this age (Table 2).

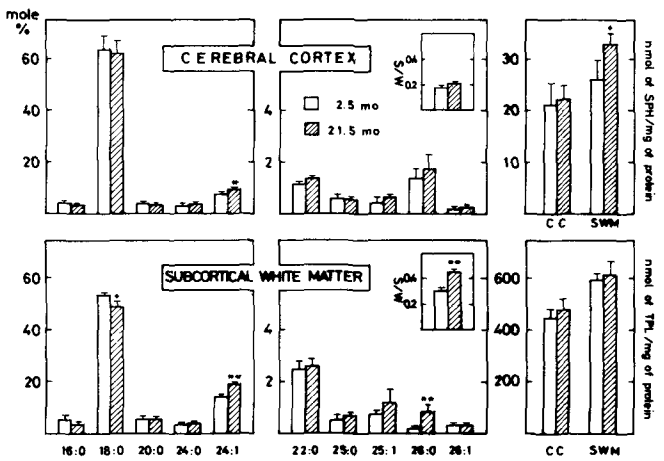

FIG. 2. Content and fatty acid composition of sphingomyelin in cerebral cortex (CC) and subcortical white matter (SWM) from adult and aged rats. Results are presented as mean values ± SD from 5–4 samples analyzed separately from control (2.5 mo) and aged (21.5 mo) rats, respectively. Each sample corresponding to 2.5- and 21.5-month-old rats was prepared with CC or SWM from two rats. Data are compared to controls using the Student's *t*-test. +, $P < 0.05$; *, $P < 0.025$; **, $P < 0.001$; M/S, monoenoic-to-saturated FA ratio.

In adult rats, the proportion of fatty acids with carbon chain lengths of 22 or more carbon atoms ranged from 18.2 to 44.6% of the total fatty acids in the brain regions studied. When the chain lengths were analyzed in cerebral cortex and subcortical white matter, the latter showed longer acyl chains than the former (Table 2).

As the age of the rats increased, the chain length of fatty acids increased. The content of acyl groups with carbon chain lengths of 22 or more carbon atoms increased 1.3-, 1.6- and 1.5-fold in 21.5-month-old rats with respect to adult rats in cerebral hemispheres, cerebellum and medulla oblongata plus pons, respectively. At 26.5 months, the content of these acyl groups increased 7.2-, 7.1- and 2.6-fold, respectively, with respect to adult animals in the regions studied.

The sum of fatty acids longer than 26 carbon atoms (C_{27} and C_{28}) represented less than 1 mole % of the total in 2.5- and 21.5-month-old rats, except in cerebral hemispheres and cerebellum, where these fatty acids increased to 1.25 and 2.08 mole %, respectively, in rats at 26.5 months.

The chain length of some fatty acids of glycolipids was shown to increase with age (25). Long-chain fatty acids with carbon chain lengths greater than 22 also have been shown to increase in patients suffering from inherited abnormalities (26) in which a deficiency in the β-oxidation of saturated and monounsaturated very long-chain fatty acids, a reaction now thought to be located exclusively in peroxisomes, takes place (27,28). Phosphatidylethanolamines and ethanolamine plasmalogens have been shown to have a lower turnover in brains of 24-month-old mice than in those of 5-month-old mice, with the half-life of the latter phospholipid being more affected than that of the former (29,30). Current evidence suggests that the respective enzymes of ether lipid metabolism are located in peroxisomes (31).

TABLE 2

Acyl Group Contents of Central Nervous System Sphingomyelin from Adult and Aged Rats[a]

	nmol/mg protein				
	SFA	MUFA	C_{22}-C_{28} FA	C_{22}-C_{28} SFA	C_{22}-C_{28} MUFA
Cerebral hemispheres					
2.5 mo	19.53 ± 6.20	4.11 ± 1.30	4.30 ± 1.40	1.64 ± 0.50	2.66 ± 0.81
21.5 mo	18.37 ± 0.86	5.03 ± 0.24	5.49 ± 0.26	1.85 ± 0.08	3.64 ± 0.17[b]
26.5 mo	43.86 ± 1.63[c]	20.71 ± 0.77[c]	31.02 ± 1.15[c]	11.25 ± 0.42[c]	19.77 ± 0.73[c]
Cerebral cortex					
2.5 mo	18.34 ± 5.68	2.96 ± 0.92	4.35 ± 1.35	2.33 ± 0.72	2.02 ± 0.63
21.5 mo	18.71 ± 2.67	3.68 ± 0.53	5.22 ± 0.75	2.63 ± 0.38	2.58 ± 0.37
Subcortical white matter					
2.5 mo	20.76 ± 4.13	5.43 ± 1.08	7.59 ± 1.51	2.93 ± 0.58	4.66 ± 0.93
21.5 mo	24.32 ± 1.83	8.98 ± 0.67[c]	11.78 ± 0.88[c]	4.01 ± 0.30[d]	7.78 ± 0.58[c]
Cerebellum					
2.5 mo	19.65 ± 3.41	6.26 ± 1.08	6.88 ± 1.19	2.87 ± 0.50	4.01 ± 0.69
21.5 mo	20.12 ± 3.91	7.66 ± 1.49	10.74 ± 2.08[d]	3.91 ± 0.76[b]	6.82 ± 1.33[c]
26.5 mo	59.28 ± 3.80[c]	34.02 ± 2.18[c]	49.11 ± 3.15[c]	15.66 ± 1.01[c]	33.47 ± 2.15[c]
Medulla oblongata + pons					
2.5 mo	25.59 ± 9.96	11.90 ± 4.63	16.72 ± 6.51	5.79 ± 2.26	10.92 ± 4.25
21.5 mo	27.38 ± 7.92	18.22 ± 5.27	24.28 ± 7.03	7.52 ± 2.17	16.76 ± 4.85
26.5 mo	37.58 ± 4.43[c]	32.91 ± 3.88[c]	43.98 ± 5.19[c]	12.31 ± 1.45[c]	31.67 ± 3.74[c]

[a]The amounts of fatty acids from sphingomyelin are given in nmol/mg protein. Results are presented as mean values ± SD from a number of animals as indicated in Table 1 and Figure 2. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; FA, fatty acids.
[b,c,d]Data are compared to adult rats using the Student's *t*-test ([b]$P < 0.05$; [c]$P < 0.005$; [d]$P < 0.025$).

Sphingomyelin synthesis was studied in minced cerebral grey matter and cerebellum of 3- and 21.5-month-old animals as a function of incubation time. [^{3}H]Choline was actively incorporated into lipids of cortical grey matter fragments from adults and aged rats. The precursor was mainly incorporated into phosphatidylcholine (PC) and, to a minor extent, into lysophosphatidylcholine and SPH. Figure 3 shows that the SPH of aged rats incorporated [^{3}H]choline to the same extent as the SPH of adult rats. However, the SPH/PC ratio was increased by 73 and 93% at 60 and 90 min of incubation, respectively, in cerebral cortex of 21.5-month-old rats as compared to that of 3-month-old rats, showing that [^{3}H]choline incorporation into PC was inhibited by aging.

Cerebral cortex and cerebellum were incubated with [^{14}C]serine to label the sphingoid base backbone. The precursor was mainly incorporated into phosphatidylserine, followed by phosphatidylethanolamine and SPH. Figure 3 shows that the pattern of serine incorporation into SPH of aged cerebral cortex was quite similar to that of adult tissue. However, the level of incorporation into SPH of cerebellum from aged rats was twice that of adult values at 60 and 90 min of incubation.

The labeling of SPH by [^{3}H]palmitic acid was similar in cerebral grey matter and cerebellum from aged rats to that in adult animals. These data are in accordance with previous findings on the effect of aging on glycerolipid synthesis of rat retina, used as a model for the central nervous system (3,32).

Our data show that *in vitro* incorporation of different

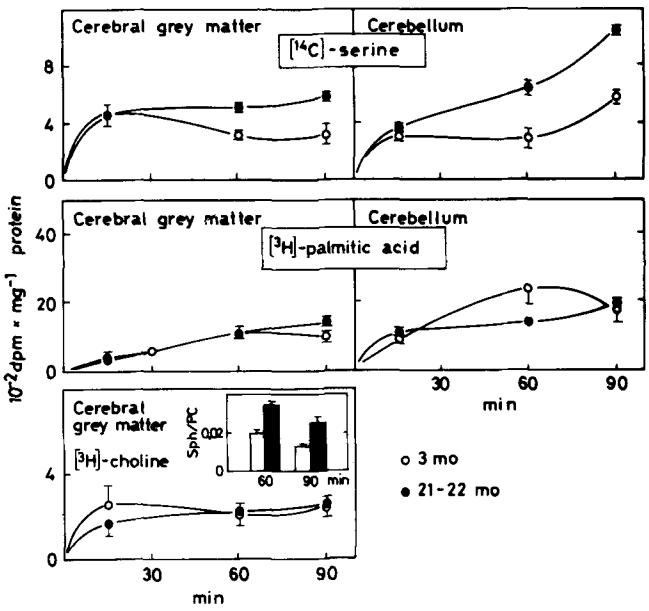

FIG. 3. Incorporation of different precursors into sphingomyelin in cerebral grey matter and cerebellum from adult (○) and aged (●) rats. Samples from cerebral grey matter and cerebellum of 3 and 21.5-month-old rats were prepared as described in Materials and Methods. Samples were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) for 15, 30 and 90 min at 37°C using 5 μCi of [^{14}C]serine or [^{3}H]choline or 20 μCi of [^{3}H]palmitic acid in a final volume of 0.2 mL. Results for each time are presented as mean value ± SD from four individual samples. Each sample was prepared with cerebral grey matter or cerebellum from two rats.

precursors, used to label the sphingoid backbone, was not affected by aging. This finding is quite surprising since RNA and lipid synthesis and turnover generally decrease with age in the central and peripheral nervous system (1,33). However, in agreement with our findings, no differences in cholineglycerophospholipid biosynthesis were observed in glial-enriched cell fractions from the brain of aged rats (34) and in aged rat retina (3,32).

In summary, our results demonstrate that [^{3}H]palmitic acid and [^{3}H]choline are incorporated *in vitro* into SPH of the central nervous system of aged rats to the same extent as they are incorporated into the SPH of adult rats; however, the labeling of SPH by [^{14}C]serine from cerebellum of aged rats was higher than in adult rats. In addition, an increase in the content of fatty acids with a carbon chain length of 22 or more carbon atoms and in the content of monoenoic fatty acids in SPH were observed. These results suggest that an altered metabolic turnover of different molecular species of SPH may be taking place and/or that the Δ9 desaturation system may be stimulated by aging.

ACKNOWLEDGMENTS

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METHOD

Determination of β-Carotene in Plasma, Blood Cells and Buccal Mucosa by Electrochemical Detection

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The β-carotene concentrations in plasma, blood cells and buccal mucosal cells were determined by high-performance liquid chromatography with electrochemical detection. This method was 1,000 times more sensitive than the conventional spectrophotometric method. Polymorphonuclear cells and red blood cells had lower β-carotene levels than the other cells. After oral administration of 580 mg/day of all-*trans* β-carotene to human male volunteers for a week, the β-carotene concentrations in all cell types increased at least several times above the original levels. ***Lipids 27,*** **840–843 (1992).**

The risk of developing cancer has been reported to be inversely correlated with the dietary intake of β-carotene (1–3), which is the major precursor of vitamin A among carotenoids. β-Carotene in part converts to vitamin A in the intestinal mucosa (4,5), while most of it is absorbed and remains in the circulation. There have been some reports (6–8) on the transport of β-carotene after ingestion in humans. Although β-carotene is known to be as efficient a singlet oxygen quencher as is lycopene (9,10), to our knowledge there have been few reports (11–13) on the physiological effects of β-carotene in association with antioxidants. Also, few reports have appeared on the β-carotene levels in body cells (14–16), which are quite low for being measured by conventional methods. Several recent studies have demonstrated the advantages of high-performance liquid chromatography (HPLC), coupled with electrochemical detection (ECD), for the determination of tocopherols (17,18), ascorbic acid (19) and thiols (20). In the present study, we were able to determine very low levels of β-carotene in biological samples by using HPLC coupled with ECD. Furthermore, changes in β-carotene levels were investigated after administration of a high-dose of oral β-carotene to adult humans.

MATERIALS AND METHODS

Sample preparation. Three healthy male volunteers (age 25 to 38) were enrolled in this study and given 580 mg of all-*trans* β-carotene/day for one week. All-*trans* β-carotene was a gift from Nippon Roche Ltd. (Tokyo, Japan). None of the volunteers took any other vitamin supplements or medication during the study. The β-carotene levels in red blood cells (RBC), plasma, platelets, mononuclear cells (MN), polymorphonuclear cells (PMN) and buccal mucosal cells (BMC) were analyzed before and at the end of β-carotene administration. Heparinized blood was collected after an overnight fast. RBC, plasma and platelets were obtained by the method reported previously (18). MN and PMN were separated by density-gradient centrifugation using "monopoly resolving medium®" (ICN Biomedicals, Asse-Relegem, Belgium) (21). The cells were then washed three times with normal saline before being used for the β-carotene analysis.

BMC were collected by gently scraping off the buccal mucosa with a spatula (18). The scraped-off cells were suspended in normal saline, washed three times with normal saline and resuspended in distilled water. The suspension was vortexed and then sonicated at 20 KHz for 30 s. Aliquots of the suspension were taken separately for the β-carotene and protein assays (22).

The protocol of the study was approved by the Ethics Committee of the hospital and was performed after informed consent was obtained from each of the subjects.

Analysis of β-carotene. One mL of ethanol containing 0.15% butylated hydroxytoluene (BHT) was added to 0.4 mL of plasma or cell suspension, followed by vigorous shaking in a nitrogen atmosphere. Five mL of *n*-hexane was added to this mixture, which was then centrifuged at 3,000 rpm for 10 min; then 4 mL of the hexane layer was evaporated under a stream of nitrogen. The residue was dissolved in 50 μL of ethanol, and a 20-μL aliquot was injected into the HPLC column. For the separation, an Irika Σ 871 HPLC instrument (Irika Co. Ltd., Kyoto, Japan) with a Vydac reverse phase C_{18} column (250 × 4.6 mm; Vydac, Hesperia, CA) was used; the detector was an Irika Σ 875 amperometric detector. The standard eluent was methanol/acetonitrile (95:5, vol/vol), which included 50 mM $NaClO_4$, and the flow rate was 1 mL/min. Authentic β-carotene was obtained from Nippon Roche K.K. (Tokyo, Japan) and dissolved in ethanol for use as standard. The purity of the standard was checked by HPLC; at least 98% of the detectable carotenoid was eluted as a single fraction. The concentration of the authentic β-carotene standard was determined with a Hitachi U-2000 spectrophotometer (Hitachi Co. Ltd., Tokyo, Japan) using a molecular coefficient $E^{\%}_{cm} = 2620$ at 453 nm in ethanol.

RESULTS AND DISCUSSION

Method of β-carotene determination. ECD is possible only for substances supporting an electrolytic reaction within an applied voltage range. A typical chromatogram of an extract of plasma obtained from a volunteer without β-carotene supplementation is shown in Figure 1 (top). The eluent was methanol/acetonitrile (95:5, vol/vol) with 50 mM $NaClO_4$. The arrow shows the putative β-carotene peak with a retention time identical to that of an authentic β-carotene standard. The voltamogram of plasma β-carotene was consistent with that of the standard β-carotene, as shown in Figure 2; both were oxidized at 0.6 V. When the eluent was changed, the presumed β-carotene peak moved in accordance with the mobility of the stan-

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Abbreviations: BHT, butylated hydroxytoluene; BMC, buccal mucosal cell; ECD, electrochemical detection; HPLC, high-performance liquid chromatography; MN, mononuclear cell; PMN, polymorphonuclear cell; RBC, red blood cell.

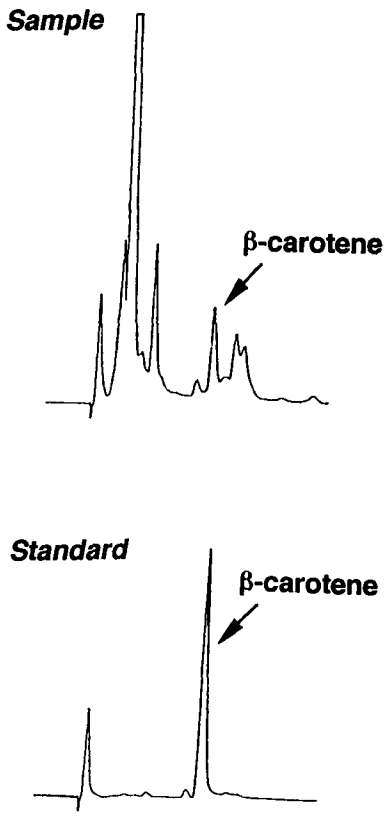

FIG. 1. Typical chromatograms of plasma and standard β-carotene. The arrows point at the β-carotene peaks. The β-carotene peaks were symmetrical and well separated from other peaks.

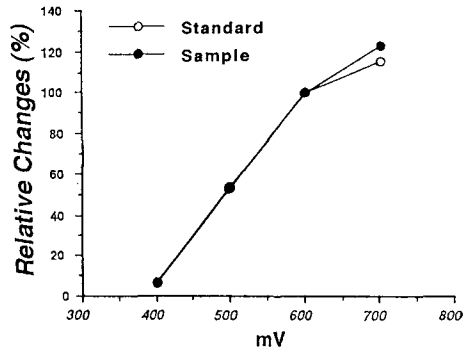

FIG. 2. Voltamograms of sample and standard β-carotene. The voltamogram curve of standard β-carotene was consistent with that of plasma; both were oxidized at 0.6 V. The X-axis shows the relative changes in current against those at 0.6 V.

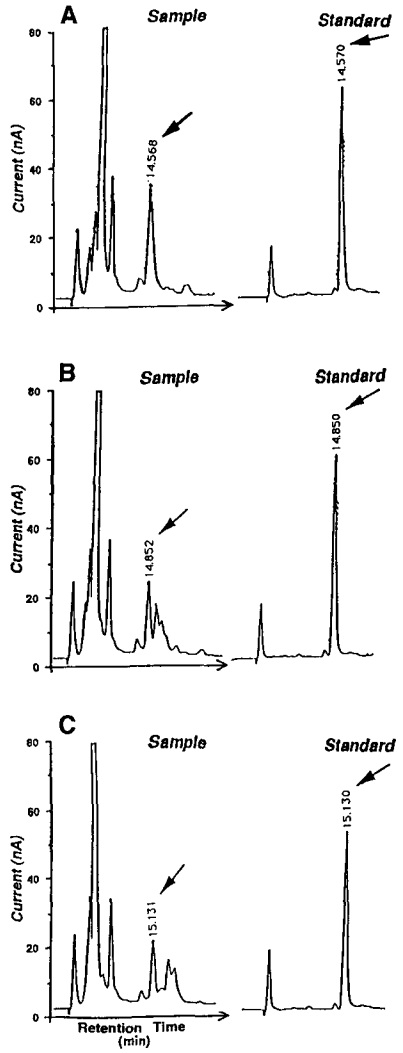

FIG. 3. Effects of eluent composition on the mobility of β-carotene in high-performance liquid chromatography. Panel A shows a chromatogram of plasma when the eluent was composed of methanol/acetonitrile (80:20, vol/vol) with 50 mM $NaClO_4$. The presumed β-carotene peak was not separated from other peaks. With an increase in the methanol concentration of the eluent (B; 90:10, C; 95:5), the surrounding peaks moved and the β-carotene peak appeared as a single symmetrical peak. The peak also moved in accordance to the mobility of the standard β-carotene.

dard β-carotene (Fig. 3). Based on this, the putative β-carotene peak was established as being due to β-carotene.

The concentration and peak area correlated in linear manner throughout the β-carotene concentration range from 0 to 100 ng. The detection limit for β-carotene (a peak area greater than 10 times the background) was 15 pg, making this assay 1,000 times more sensitive than previous methods based on spectrophotometric detection (Fig. 4). The sensitivity was sufficient to determine β-carotene levels in various biological samples.

Five replicate samples from pooled plasma were analyzed. The mean concentration was 0.195 μg/mL, and the intra-batch coefficient of variation was 3.6%, as shown in Table 1. When the recovery test was done for this assay, a satisfactory 95.3% recovery was measured as shown in Table 2.

Determination of cellular β-carotene levels. We measured the concentrations of β-carotene in plasma, blood cells and BMC from volunteers which did not receive a supplement of β-carotene. Typical chromatograms are shown in Figures 1 and 5. Although the β-carotene levels of these samples were very low, our assay was sensitive enough to detect them. When conventional detection was used for the red blood cell (14) and leukocyte (15) assays, β-carotene could be detected, but it could not be quantified.

The β-carotene concentrations in the cells and in plasma from the three male volunteers are shown in Table 3. After oral administration of 580 mg of β-carotene/day for one

METHOD

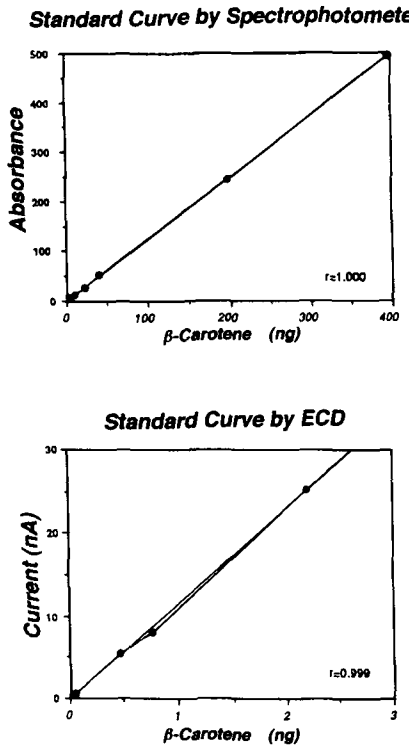

FIG. 4. Calibration curves for standard β-carotene by electrochemical detection (ECD) and spectrophotometric detection. The limit of detection by the spectrophotometric method was 15 ng of β-carotene, and that for ECD was 15 pg.

TABLE 1

Intra-Batch Coefficient Variance of β-Carotene in Plasma Samples

β-Carotene in plasma pool samples (μg/mL)				
1	2	3	4	5
0.190	0.187	0.198	0.205	0.196

Mean	0.195 μg/mL
SD	0.007 μg/mL
CV[a]	3.612%

[a]CV, coefficient variance.

TABLE 2

Recovery of β-Carotene in Plasma Pool Samples

β-Carotene in plasma (μg/mL)	Added β-carotene (μg/mL)	Observed β-carotene (μg/mL)	Recovery[a] (%)
0.190	0.052	0.230	95
0.188	0.055	0.230	95
0.193	0.108	0.280	93
0.202	0.112	0.278	89
0.186	0.158	0.348	101
0.192	0.161	0.348	99

[a]Average 95.3.

week, the β-carotene concentrations in plasma, platelet, BMC, MN, PMN and RBC increased to 35-, 6-, 8-, 4-, 3- and 7-fold, respectively. The difference in incremental changes in β-carotene levels are likely to depend on variations in lifespan, turnover and lipid content of the cells. Further studies are needed to determine the factors that influence the incremental changes in β-carotene levels in biological samples.

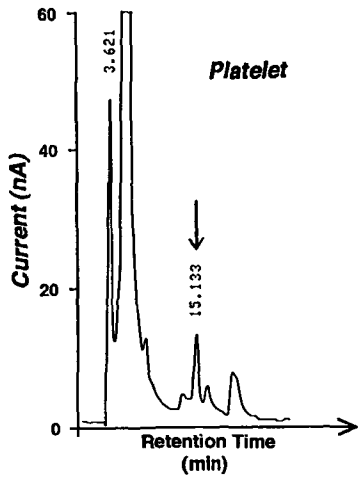

FIG. 5. Typical chromatogram of a platelet sample. The arrow points at the β-carotene peak. β-Carotene also was detected in red blood cells, mononuclear cells and buccal mucosa.

TABLE 3

Changes in the β-Carotene Concentration in Various Biological Samples after Oral β-Carotene Administration[a]

	Before	After	
Plasma	0.19 ± 0.03	6.85 ± 0.16	μg/mL
Platelets	5.05 ± 1.26	37.30 ± 7.70	ng/mg protein
BMC	0.79 ± 0.26	6.23 ± 0.84	ng/mg protein
MN	0.71 ± 0.28	2.68 ± 0.66	$\mu g/10^9$ cells
PMN	0.20 ± 0.09	0.58 ± 0.11	$\mu g/10^9$
RBC	1.51 ± 0.74	10.2 ± 8.5	ng/mL packed cells

[a]Values are mean ± SEM (n = 3). Abbreviations: BMC, buccal mucosal cells; NM, mononuclear cell; PMN, polymorphonuclear cell; RBC, red blood cell.

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COMMUNICATION

Gender and Dietary Fat Affect α-Tocopherol Status in F344/N Rats

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For four weeks, groups of eight male and eight female F344/N rats were fed diets containing 15.5, 20, 30 or 40% of energy (en%) as fat. The fat was composed of corn oil and beef tallow with 9 en% from linoleate in all diets. Females had greater mean hepatic α-tocopherol levels, whereas males had greater plasma α-tocopherol and cholesterol concentrations. In males, the plasma ratio of α-tocopherol/cholesterol was significantly greater than in females ($P < 0.05$). Plasma α-tocopherol increased with increasing en% fat ($r = 0.51$, $P < 0.001$) in both sexes, but dietary fat did not alter hepatic α-tocopherol levels. These results suggest that plasma α-tocopherol may serve as a biomarker of total dietary fat intake and that in F344/N rats gender differences affect α-tocopherol and cholesterol status.
Lipids 27, 844-846 (1992).

Oxidative stress may underlie tumor promotion since many tumor-promoting agents, such as phenobarbital and 12-*O*-tetradecanoyl phorbol acetate stimulate oxidative stress (1-3). Antioxidants suppress growth of preneoplastic cells (4) and inhibit promotion-associated cellular transduction (5) but may, like α-tocopherol, be depleted during oxidative stress (6).

Increased or altered dietary fat concentrations promote cancer development, which may be due to increased oxidative stress (7,8). Total dietary fat, by stimulating elongation, desaturation and deposition of fatty acids, can influence the tissue content of oxidizable lipids. Because α-tocopherol functions as an antioxidant *in vivo*, increased dietary fat may stimulate α-tocopherol depletion and result in increased oxidative stress and cancer susceptibility.

In two model systems, female rats have been reported to be more susceptible to the promotion of hepatocarcinogenesis than males (9,10). The mechanism underlying the greater susceptibility of the female to the promotion of hepatocarcinogenesis is unknown but may be related to susceptibility to oxidative stress. Therefore, gender differences in response to dietary fat may be an important determinant of cancer development. This study was designed to determine to what extent gender and fat calories alter an animal's susceptibility to oxidative stress, as reflected in hepatic and plasma α-tocopherol status.

MATERIALS AND METHODS

Animals and diets. Thirty-two male and thirty-two female F344/N rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) at eight weeks of age. They were housed in a room maintained at 22°C and approximately 50% humidity. Four diets were fed which contained 15.5, 20, 30 or 50 en% (percentage of energy) fat (Table 1). Each diet provided 9% of energy (en%) as linoleate and was prepared from corn oil and beef tallow. Diets were designed to provide equal amounts of amino acids, vitamins and minerals per calorie and 4.0-4.6 cal/g diet. After four weeks, rats were anesthetized with diethyl ether and blood was obtained from the jugular vein. Animals were killed by ether inhalation and 0.5 g minced liver was homogenized from each rat in 5 mL of 50 mM potassium phosphate buffer adjusted to pH 7.4. Tissue and plasma samples were frozen on dry ice and stored at −80°C for later analysis.

TABLE 1

Diet Composition

Ingredient (g/kg)	Energy percentage as fat			
	15.5	20	30	40
Beef tallow[a]	—	23.6	77.8	139.6
Corn oil[b]	68.7	67.7	67.5	66.5
Casein[c]	193.4	198.5	210.5	224.1
Corn starch[a]	443.3	407.9	323.7	228.5
Dextrose[a]	193.4	198.5	210.5	224.1
Cellulose[a]	48.4	49.6	52.6	56.0
AIN vitamin mix 76[a]	9.7	9.9	10.5	11.2
AIN mineral mix 76[a]	23.9	34.7	36.8	39.2
$CaCO_3$[a]	4.4	4.5	4.7	5.0
Choline bitartrate[a]	1.9	2.0	2.1	2.2
L-methionine[a]	2.9	3.0	3.2	3.0
Ascorbate[d]	0.1	0.1	0.1	0.1

[a]Teklad (Madison, WI).
[b]Iowa State University Food Stores (Ames, IA).
[c]United States Biochemical Co. (Cleveland, OH).
[d]Sigma Chemical Co. (St. Louis, MO).

α-Tocopherol and cholesterol analyses. α-Tocopherol content was determined in both plasma and liver by the method of Catignani and Bieri (11). All samples were processed in a room illuminated with yellow light. Samples were mixed with internal standard (α-tocopheryl acetate in ethanol) and extracted two times with hexane. A portion of the hexane extract was evaporated under nitrogen and the residue redissolved in methanol. A portion of the methanol solution was injected into a high-pressure liquid chromatography (HPLC) system which consisted of a Beckman pump (Model 110A, Palo Alto, CA) with solvent injector (Rheodyne, Cotati, CA), a Hitachi ultraviolet (UV)/visible spectrophotometer (Model 110-10, Tokyo, Japan) set at 290 nm, Beckman recorder (Model 155-00) and a Hewlett-Packard integrator (Model 3390A, Avondale, PA). Reversed-phase HPLC was carried out on a Waters "Resolve" 5 μm C-18 column (Milford, PA) equipped with an Upchurch guard column (Oak Harbor, WA). The mobile phase was 100% methanol and the flow rate was 2.0 mL/min. Plasma cholesterol concentrations were

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Abbreviations: en%, percentage of energy; HDL, high density lipoproteins; HPLC, high-pressure liquid chromatography; LDL, low density lipoproteins; UV, ultraviolet.

measured by an enzymatic method (12) (Kit No. 352, Sigma Chemical Co., St. Louis, MO).

Statistical analysis. Each sample analysis was performed in duplicate. Statistical analysis was done with the Pearson Correlation Coefficient procedure of the Statistical Analysis System, version 5.16 (SAS, Cary, NC). Student's *t*-tests were used to determine the least significant difference between means. Two-way analysis of variance was used to determine the interaction between en% fat and sex.

RESULTS AND DISCUSSION

Plasma α-tocopherol concentrations were significantly greater in males than in females, whereas hepatic α-tocopherol concentrations were significantly greater in females than in males (Table 2). Plasma α-tocopherol and hepatic α-tocopherol were negatively correlated ($r = -0.30$, $P < 0.01$). Plasma total cholesterol concentrations were not affected by diet but were significantly elevated in males as compared with females ($P < 0.05$), as was the plasma ratio of mg α-tocopherol to g cholesterol.

Few previous studies have assessed the effects of gender on α-tocopherol status. In a recent cross-sectional study in adolescents which examined fat-soluble vitamin status, males with certain indices of maturation demonstrated higher plasma α-tocopherol concentrations than females. However, this effect disappeared after adjustment for plasma cholesterol concentrations (13). In gray seals, lactating females were found to have significantly lower concentrations of vitamin E than males, whereas no differences in serum vitamin E were found in juveniles of the species (14). In humans, the main carriers of α-tocopherol are the low density lipoproteins (LDL) and high density lipoproteins (HDL), and more α-tocopherol has been found in LDL than HDL in males, with the opposite distribution in females (15). Therefore, male hepatic α-tocopherol may be diminished and plasma levels enhanced by lipoprotein export (especially LDL), because males of a species generally have greater plasma LDL levels than females (16). Our study suggests that under carefully controlled feeding regimens, gender differences do exist in F344/N rats that may affect α-tocopherol transport and storage.

The correlation between increasing dietary fat and increasing plasma α-tocopherol ($r = 0.51$, $P < 0.0001$) and the significant increase in plasma α-tocopherol when 40 en% fat was fed compared with 20 en% fat suggest that increasing dietary fat stimulated α-tocopherol transport.

TABLE 2

Effect of Gender on α-Tocopherol and Cholesterol Status[a]

Sex[b]	Plasma α-tocopherol (μg/mL)	Plasma total cholesterol (mg/mL)	Plasma α-tocopherol/ cholesterol (mg/g)	Hepatic α-tocopherol (μg/g)
M	8.7 ± 1.4[d]	72.8 ± 8.1[d]	12.2 ± 1.4[d]	37.8 ± 7.8[c]
F	6.1 ± 1.4[c]	54.6 ± 4.9[c]	11.2 ± 2.2[c]	51.0 ± 8.3[d]

[a]Values are means ± standard deviations. Groups that do not share the same letter are significantly different from one another, $P < 0.05$.

[b]n = 32 for either males or females.

TABLE 3

Effect of Fat Intake on α-Tocopherol and Cholesterol Status[a,b]

Diet (en% fat)	Plasma α-tocopherol (μg/mL)	Plasma total cholesterol (mg/mL)	Plasma α-tocopherol/ cholesterol (mg/g)	Hepatic α-tocopherol (μg/g)
15.5	7.2 ± 2.0[d,c]	66.5 ± 12.2	10.7 ± 1.8[c]	42.9 ± 10.5
20	7.0 ± 1.9[c]	66.2 ± 12.2	11.2 ± 1.8[c]	44.5 ± 10.9
30	7.9 ± 2.3[d,c]	62.3 ± 12.0	12.5 ± 2.3[d]	46.7 ± 10.6
40	8.1 ± 1.4[d]	62.6 ± 9.1	12.6 ± 0.9[d]	43.6 ± 10.3

[a]Values are means ± standard deviations. Groups that do not share the same letter are significantly different from one another, $P < 0.05$.

[b]n = 16 for each dietary group.

It is possible that greater fat intake stimulated absorption of fat-soluble vitamins (17). However, plasma α-tocopherol concentrations did not reflect liver α-tocopherol status (Table 3). Petersson *et al.* (18) also showed that increased plasma α-tocopherol concentrations were not associated with increased hepatic α-tocopherol content. Perhaps greater en% fat diets increased hepatic export of α-tocopherol as increasing dietary fat, especially saturated fat, increases plasma LDL (19).

Plasma α-tocopherol concentration may be a useful biomarker of human dietary fat intake and an important tool in monitoring compliance with modified fat diets. Plasma α-tocopherol levels are increased when 10% lard is fed, compared with 10% corn oil, and both oils are stripped of vitamin E (20). Human subjects given α-tocopherol supplements had greater plasma α-tocopherol levels when fed a high fat (43 en%) diet than when fed a low fat (25 en%) diet (21). Five out of eight adult males fed a diet containing beef fat for 5 d after 7 mon of corn oil feeding showed increased plasma tocopherol levels (22).

Differences, such as those observed in α-tocopherol status due to gender and dietary fat content, may be mediated by mechanisms which involve α-tocopherol absorption and transport and are related to lipoprotein composition. Carnivorous species, in general, have greater plasma α-tocopherol than herbivorous species (23), and the influence of dietary fat source (animal *vs.* plant) and fatty acid composition on plasma α-tocopherol status is an important point for further study.

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SYMPOSIUM

Milk Lipids

A Symposium held at the AOCS Annual Meeting, Baltimore, MD, April 1990

Opening Remarks

Research on milk lipids, which languished in the seventies, has had a renaissance in the eighties. Most of the recent interest in this field has been focussed on the lipids of human milk. In fact, there have been more papers published on human milk lipids during the past ten years than on milk lipids of bovine origin. More recently, researchers have also analyzed the milk of various mammals with the implicit goal that animal models applicable to the human situation would be found.

The metabolism of $\omega 6$ and $\omega 3$ fatty acids, primarily in human milk, has become a fertile area of research, and many intriguing questions remain to be answered. Also, as various non-nutritive roles of lipids in human milk are being recognized, *e.g.*, the inhibition of enterotoxins by gangliosides, these functions of milk deserve further study. It is also astonishing that only a few fatty acid analyses of bovine milk lipids have been done since gas-liquid chromatography capillary columns have been available. Most fluid dairy products consumed in the United States today are homogenized by processes which totally alter the membrane surrounding the fat globule which, in turn, can be expected to alter digestion. Yet, there are no data addressing these questions. Clearly, additional research efforts and support are needed to carry out further investigations.

I hope that the papers presented in this symposium will stimulate advanced research on milk lipids and generate the funding required to support these urgently needed efforts.

Robert G. Jensen

REVIEW

Human Milk in Disease: Lipid Composition[1]

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Differences in the lipid composition of human milk have been described in maternal diseases known to affect fat metabolism. Diseases such as diabetes, cystic fibrosis, hypobetalipoproteinemia and Type I hyperlipoproteinemia affect the quantity and quality of human milk fat. Increased fatty acid chain elongation and changes in desaturation (especially Δ6 desaturase), as well as changes in lipid class composition, have been shown in diabetes and cystic fibrosis, whereas compensatory increases in medium-chain fatty acids have been described in hypobetalipoproteinemia and Type I hyperlipoproteinemia. It is important to realize that these observations were made either on single women or on very small groups of women. In infant diseases, such as breast milk jaundice and ectopic eczema, changes in polyunsaturated fatty acids in maternal milk have been described.

Lipids 27, 848–857 (1992).

Human milk is the recommended food for the newborn infant (1,2). The increase in breastfeeding in America (3) has paralleled our growing understanding of the composition of human milk (4,5) and of its specific function in the newborn (5–7). As a result of the increasing knowledge of the beneficial effects of human milk for the newborn infant, women with chronic metabolic diseases now elect to breastfeed their children. However, although information is available on changes of milk composition as a result of some diseases, such as mastitis (8,9), little is known about the effects of metabolic diseases on human milk. In this report we are reviewing information about milk lipid composition in disease.

Lipids are essential for infant growth not only because they provide more than 50% of the energy requirements and are vehicles for fat soluble vitamins, but also because they are needed for brain development, are precursors of prostaglandins and hormones, and are essential constituents of all cell membranes. Lipids are the most variable component of milk, their amounts changing as a function of length of lactation (*i.e.*, lower concentrations in colostrum than in mature milk), as well as changing within the day (diurnal) and within each individual feed (low in fore and high in hind milk) (10). Furthermore, milk fat composition is markedly affected by maternal nutrition (10) as well as by length of gestation (10,11), parity (12) and other modulating factors. In established lactation, however, the amount of fat secreted into milk is independent of nutritional intake and is constant for individual women. It was therefore of interest to assess whether maternal diseases known to affect lipid metabolism might also affect the content and composition of the fat secreted into milk. To date there are not many studies that have investigated this topic. Thus, most of the data are derived from studies of single patients or of small numbers of women suffering from the same disease. The studies that we summarize here are of either maternal diseases, such as diabetes, cystic fibrosis, hypobetalipoproteinemia, type I hyperlipoproteinemia; or diseases of the infant, such as atopic eczema or breast milk jaundice.

Composition and origin of milk fat. The fat in milk is contained within membrane-enclosed milk fat globules. The core of the globules consists of triglycerides (98–99% of total milk fat), whereas the globule membrane is composed mainly of phospholipids, cholesterol and proteins. The triglycerides are composed chiefly of long-chain fatty acids (up to 90% of fatty acids in mature human milk) derived from the circulation. These long-chain fatty acids reach the mammary epithelial cells after their release from lipoprotein-triglyceride at the capillary endothelium by the enzyme lipoprotein lipase (13–15). Short- and medium-chain fatty acids (C_8–C_{16}) in human milk are synthesized by the fatty acid synthetase thioesterase II Complex (13,16). Phospholipids are synthesized *de novo* within the mammary gland, whereas cholesterol originates from *de novo* synthesis and from the circulation. The large body of literature on the topic of human milk lipids has been reviewed recently (10). The amount and fatty acid composition of milk fat are the final product of the processes of fat digestion, absorption, transport in the circulation, release from adipose tissue storage and, finally, uptake into the lactating mammary gland for long-chain fatty acids, as well as *de novo* biosynthesis for medium-chain fatty acids. Therefore, diseases that affect any of these processes might also affect the fat of human milk.

Diabetes

Diabetes affects many aspects of lipid metabolism. Amount and composition of milk lipids have been studied by several investigators at early lactation only (17) and up to three months of lactation (18–20). The total amount of milk fat is within the normal range in diabetic woman (Table 1). There are conflicting data on the concentration of medium-chain fatty acid (C_{10}-C_{14}) in the milk of diabetic women. Significantly lower than normal levels of medium-chain fatty acids were reported in the milk of one diabetic woman studied at the onset of lactation (17) and in the milk of three diabetic women studied during the first three months of lactation (19) (Table 2). However, when the number of subjects studied was increased from 3 to 21, significantly higher medium-

TABLE 1

Milk Lipids in Diabetes: Total Fat

Study (reference)[a]	Subjects D/C[b]	Lactation days	Total fat (g/dL)	
			D	C
1 (17)	1/13	6–7	2.95	4.53
2 (18)	2/12	42	3.60	4.40
3 (19)	3/3	7–84	3.30	2.82
4 (21)	7/7	7–84	3.03	3.16

[a]Study number in bold, reference number in parentheses.
[b]D, diabetes; C, control.

[1]Based on a paper presented at the Symposium on Milk Lipids held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

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Abbreviations: CF, cystic fibrosis; EFA, essential fatty acid; FA, fatty acid(s); FFA, free fatty acid(s); MCFA, medium-chain fatty acid(s); PUFA, polyunsaturated fatty acid(s); TG, triglyceride(s).

TABLE 2

Milk Lipids in Diabetes: Medium-Chain Fatty Acids (MCFA)

Study (reference)[a]	Subjects D/C[b]	Lactation days	MCFA% Diabetes[c]				MCFA% Control			
			10:0	12:0	14:0	Total	10:0	12:0	14:0	Total
1 (17)	1/6	6–7	0.09	1.69	3.67	5.45	1.03	5.66	7.12	13.81
2 (19)	3/3	7–84	0.43	1.92	3.30	5.65	0.58	3.05	4.05	7.68
3 (20)	21/22	14	—	—	—	13.62	—	—	—	10.58

[a]As in Table 1.
[b]D, diabetes; C, control.
[c]Total MCFA; data are means for each group.

TABLE 3

Milk Lipids in Diabetes: Cholesterol

Study (reference)[a]	Subjects D/C[b]	Lactation days	Cholesterol (% of total lipids)					
			Diabetes			Control		
			3[c]	7	2–84	3	7	2–84
1 (17)	1/6	3–84	0.39	0.16		1.30	0.70	0.40
2 (21)	7/7	2–84	—	—	0.39	—	—	0.66

[a]As in Table 1.
[b]D, diabetes, C, control.
[c]Days of lactation.

TABLE 4

Lipid Class Composition of Milk in Diabetes[a]

Class	Lactation day				
	3 (n = 2)	4 (n = 3)	5 (n = 5)	6 (n = 3)	7 (n = 2)
Monoglycerides (%)	0.35	0.39	0.31	0.18	0.12
Free fatty acids (%)	2.22	24.70	23.49	24.18	19.64
Cholesterol (%)	0.21	0.12	0.09	0.09	0.09
1,2-Diglycerides (%)	0.11	0.31	0.27	0.22	0.22
1,3-Diglycerides (%)	0.12	0.38	0.35	0.22	0.22
Triglycerides (%)	96.84	73.90	75.32	75.01	79.66
Cholesteryl esters (%)	0.18	0.21	0.16	0.10	0.07
Total neutral lipid (g/L)	9.3	16.4	25.0	23.0	29.4

[a]Reproduced from Bitman *et al.* (17).

chain fatty acid levels were found in the milk of the diabetic women at 14–84 d of lactation, as compared to that of healthy women. Jackson *et al.* (20) however, showed that in this latter study there were no differences between diabetic and control women in milk medium-chain fatty acid concentrations at 7 d *postpartum* (9.46 and 8.85% in the milk of 23 diabetic and 20 control women, respectively). The significant difference between higher medium-chain fatty acids of diabetic as compared to control milk was eliminated when gestational age was used as a covariate for both mothers of full term (>37 wk) and all subjects. Gestational age differences in the diabetic (37.6 wk) and control (39.6 wk) groups may account for the group differences. Medium-chain fatty acid concentrations were reported to be higher in the milk of women who deliver prematurely (11).

The cholesterol content of milk has been studied at the onset of lactation in one diabetic woman (17), and during the first three months of lactation in seven diabetic women (21). Cholesterol concentrations were lower at the onset as well as during established lactation in milk secreted by diabetic women when compared to appropriate control groups (Table 3). Detailed analyses of lipid classes and of fatty acid composition are only available to date on the milk from one diabetic woman studied at the onset of lactation. The main difference in lipid class composition (Table 4), in addition to lower milk cholesterol, was the much higher concentration of free fatty acids (FFA) [normally less than 0.5% (22)] that increased to 23% of total lipid on days 4–7 *postpartum*. The high FFA concentration is not due to a technical artifact since there was no relationship between milk FFA concentration and sampling, handling or storage of the milk specimens (17). The possibility that the high FFA level is due to hydrolysis of milk triglyceride by the lipoprotein lipase of milk was investigated. However, the data show no correlation between lipoprotein lipase activity level and FFA concentrations (17). It seems, therefore, that there might be impaired esterification in the mammary gland in diabetes. These observations on a single subject during a period of only four days after initiation of lactation will have to be confirmed in a large group studied throughout lactation. Com-

parison of the fatty acid composition of the milk from one diabetic woman with that of 12 healthy controls shows several marked differences. The technique of Holman and Johnson (23) in which the ratio of the value in the disease as compared to that in the control is used as an index of normalcy was applied to the evaluation of differences in milk fatty acid pattern between diabetes and control. The data presented in Table 5 show, in addition to lower levels of medium-chain fatty acids described above (Table 2), higher concentration of long-chain unsaturated (oleic and linoleic) and polyunsaturated (18:2n-6, 18:3n-3, 22:5n-6, 22:5n-3), as well as 21:0 fatty acids in the milk of the diabetic subject, as compared to the healthy controls. Although our knowledge on the changes in milk fat composition in diabetes should await more comprehensive studies in a greater number of subjects, the studies described above suggest that (i) total milk fat is within the range of healthy women; (ii) medium-chain fatty acid synthesis may be impaired (at least at early stages of lactation), whether this might be due to lower dietary carbohydrate intake or to differences of mammary gland metabolism secondary to imbalance of insulin secretion remains to be evaluated; (iii) increased chain elongation comparable to that described for serum phospholipid fatty acids of diabetic patients (23); and (iv) lower levels of milk cholesterol. Since cholesterol is a component of the milk fat globule membrane, the latter suggests possible physical changes in globule size, *e.g.*, greater diameter to permit packaging of more core triglyceride per unit membrane. Whether this might affect the stability of milk fat globules and fat digestion by the infant (24) requires further study.

Cystic Fibrosis

Cystic fibrosis is a genetic disease with highest incidence (1 in 2000) in Caucasians. Patients with cystic fibrosis with (25–28) and without (29) pancreatic insufficiency have changes in blood and tissue fatty acid composition that are consistent with essential fatty acid deficiency (23,30,31). Lower plasma and tissue levels of linoleic acid (18:2n-6) have been attributed to fat malabsorption or dietary deficiency (32–37). Decreased caloric intake and increased caloric needs in cystic fibrosis patients could lead to oxidation of linoleic acid to meet immediate energy needs and, thus, decrease the amount of linoleic acid available for arachidonic acid and prostaglandin (33) synthesis. Differences in plasma and tissue levels of linoleic acid as related to pancreatic function (lower levels in pancreatic insufficient patients) have been described (37). Altered hepatic function (36) or an enzymatic defect in fatty acid desaturation (27,28,38) also have been suggested as possible reasons for the fatty acid changes in cystic fibrosis.

We have studied the lipid composition of milk of six women with cystic fibrosis who delivered full term infants and decided to breastfeed (39). An earlier study of one woman indicated very low milk fat content (1.3 g/dL), suggesting that in cystic fibrosis, breastfeeding might not be able to meet the needs for normal infant growth (40). Be-

TABLE 5

Comparison of Fatty Acid Composition of Colostrum and Transitional Milk from Diabetic and Healthy Mothers

	Colostrum			Transitional milk		
	Diabetic[a]	Healthy[b]		Diabetic[a]	Healthy[b]	
Fatty acid	% of total		Normalcy ratio[c]	% of total		Normalcy ratio[c]
10:0	—	0.28	—	0.09	1.03	0.09
12:0	0.61	3.11	0.20	1.69	5.66	0.30
13:0	0.02	—	—	0.03	—	—
14:0	4.01	6.07	0.66	3.67	7.12	0.52
14:1n-5	0.28	—	—	0.27	—	—
15:0	0.29	0.20	1.45	0.35	0.24	1.46
16br	0.09	—	—	0.17	—	—
16:0	22.46	25.75	0.87	21.67	22.83	0.95
16:1n-7	3.29	2.97	1.11	3.01	3.10	0.97
16:2n-7/17:0	0.48	0.58	0.83	0.66	0.48	1.38
18:0	6.97	8.53	0.82	7.31	6.91	1.06
18:1n-9	39.70	34.78	1.14	38.34	34.86	1.10
19:0	0.33	—	—	0.44	—	—
18:2n-6	14.49	12.13	1.19	16.59	13.95	1.19
18:3n-6/20:0	0.28	0.09	3.11	0.39	0.10	3.90
18:3n-3/20:1	2.02	1.16	1.74	2.30	0.87	2.64
21:0	0.15	0.07	2.14	0.19	0.07	2.71
20:2n-6	1.01	0.88	1.15	0.86	0.51	1.69
20:3n-6	0.66	0.82	0.80	0.53	0.58	0.91
20:4n-6	1.11	1.15	0.97	0.84	0.82	1.02
24:0	0.20	—	—	0.11	—	—
24:4n-6	0.83	0.70	1.19	0.34	0.33	1.03
22:5n-6	0.24	0.11	2.18	0.11	0.05	2.20
22:5n-3	0.45	0.22	2.05	0.18	0.13	1.38
22:6n-3	0.13	0.36	0.36	0.11	0.30	0.37

[a]Data for diabetic mother; colostrum from *postpartum* days 4–5; transitional milk from days 6–7. Data from Bitman *et al.* (17).
[b]Data from healthy mothers are from Bitman *et al.* (11).
[c]Normalcy ratio = value in disease/value in control.

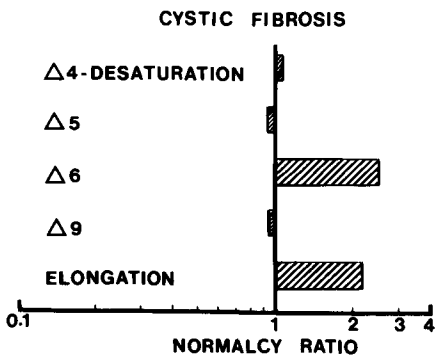

FIG. 1. Relative activities of enzyme systems involved in polyunsaturated fatty acid metabolism in cystic fibrosis milk. From Bitman *et al.* (39).

cause of changes in fatty acid profiles of plasma and tissues in cystic fibrosis, we have examined lipid distribution within neutral lipid and phospholipid classes, as well as the fatty acid composition of the total lipids of milk. The data are presented in Tables 6–9 and Figure 1. Total milk fat, although slightly lower than normal, was within the range compatible with normal infant development (Table 6). Indeed, all six mothers breastfed their infants for 3 to 13 wk and infant weight gains were normal. No major changes in neutral lipids were evident, triglycerides amounting to approximately 98% of total fat. Differences were, however, evident in phospholipid classes—milk of the cystic fibrosis patients contained similar amounts of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine (about 25% each) as compared to higher concentrations of sphingomyelin and lower concentrations of phosphatidylethanolamine in healthy human milk (Table 7). These changes might affect the milk fat globule membrane. Cholesterol levels and distribution between the free and esterified forms were similar to those in normal milk (Table 8). Fatty acid composition of total fat indicated lower levels of the essential fatty acid linoleic acid 18:2n-6, as well as of its elongation and desaturation product arachidonic acid 20:4n-6 (Table 9). Other polyunsaturated fatty acids were elevated in cystic fibrosis (Table 9). The concentrations of 16:2, 18:3 and of several long-chain polyunsaturated fatty acids were about twice those in healthy milk. Normalcy ratios (cystic fibrosis to healthy) showed that 16:2/17:0, 18:3n-3, 20:2n-6, 22:4n-6, 22:5n-6 and 22:5n-3 were all elevated in cystic fibrosis milk. The data indicate that while the enzymes responsible for Δ6 desaturation and for chain elongation were elevated, Δ4, Δ5 and Δ9 desaturation were within normal ranges (Fig. 1). The fatty acid pattern of the milk of cystic fibrosis patients showed a number of abnormalities consistent with essential fatty acid deficiency. Given the important role that linoleic acid and its metabolites have in maintaining structure (membranes) and function (prostaglandin synthesis) of cells, it may be important to supplement breastfeeding women who have cystic fibrosis with this essential fatty acid in order to provide adequate nutrition for their infants. Given the precarious nutritional status of cystic fibrosis patients and the additional caloric demand of milk production (41), it is advisable to closely monitor the nutritional status of lactating cystic fibrosis patients. This is necessary for both maternal and infant health.

TABLE 6

Lipid Class Composition of Milk from Five Mothers with Cystic Fibrosis[a]

	Cystic fibrosis milk			
	Colostrum	Transitional	Mature	Healthy mature milk
Lipid class (%)				
Monoglycerides	0.12	0.02	0.20	0
Free fatty acids	0.29	0.34	1.65	0.08
Cholesterol	0.61	0.44	0.18	0.34
1,2-Diglycerides	0.48	0.26	0.74	0.01
Triglycerides	98.38	98.81	98.18	98.76
Cholesteryl esters	0.26	0.14	0.05	
Neutral lipids (g/dL)	2.36	2.87	2.94	3.71
Lactation day	3	11	58	128
Number of mothers	5	3	4	6

[a]From Bitman *et al.* (39).

TABLE 7

Phospholipid Composition of Cystic Fibrosis Milk From Patient 1[a]

	Lactation day				
Phospholipids	3	49	63	70	Healthy milk
Sphingomyelin (%)	42.6	32.3	28.6	25.9	37.5
Phosphatidylcholine (%)	23.5	23.0	23.8	24.1	28.4
Phosphatidylserine (%)	13.6	15.0	12.7	14.8	8.8
Phosphatidylinositol (%)	5.6	7.5	11.1	11.1	6.1
Phosphatidylethanolamine (%)	14.8	22.1	23.8	24.1	19.3

[a]From Bitman *et al.* (39).

TABLE 8

Cholesterol and Cholesteryl Ester in Cystic Fibrosis Milk[a]

Lactation stage	Number of mothers	Total cholesterol (mg/dL)	Cholesterol (mg/dL)	Cholesteryl ester (mg/dL)	Ester (%)
Colostrum	6	15.78	11.13	4.65	29.5
Transitional	3	17.81	13.43	4.38	24.6
Mature	7	7.87	6.54	1.33	16.9

[a]Values for cholesterol and cholesteryl ester content in the milk, respectively, of women without cystic fibrosis (in mg/dL) were: colostrum, 23.25, 4.92; transitional milk, 13.40, 1.8; mature milk, 7.78, 092. From Bitman *et al.* (39).

TABLE 9

Fatty Acid Patterns of Mature Milk from Mothers with and Without Cystic Fibrosis[a]

Fatty acids	Mean % for women with cystic fibrosis	Mean % for women without cystic fibrosis	Pooled SE	*P* Value	Normalcy ratio
8:0	0.05	0.00	0.00	<0.005	—
18:0	1.34	0.92	0.07	<0.01	1.46
12:0	6.47	4.64	0.32	<0.02	1.39
14:0	7.19	5.80	0.38	n.s.	1.24
15:0	0.35	0.28	0.02	n.s.	1.25
16:0	22.94	22.21	0.80	n.s.	1.03
16:1n-7	3.47	3.58	0.08	n.s.	0.97
16:2n-7/17:0	1.59	0.48	0.17	<0.007	3.31
18:0	6.59	7.82	0.19	<0.007	0.84
18:1n-9	33.43	36.26	0.91	n.s.	0.92
18:2n-6	11.67	15.23	0.67	<0.02	0.77
18:3n-3	1.15	0.87	0.09	n.s.	1.32
18:3n-6/20:0	0.20	0.13	0.05	n.s.	1.54
20:2n-6	0.26	0.17	0.04	n.s.	1.53
20:3n-6	0.35	0.41	0.03	n.s.	0.85
20:4n-6	0.45	0.57	0.04	n.s.	0.79
21:0	0.19	0.09	0.02	<0.008	2.11
22:4n-6	0.61	0.11	0.08	<0.01	5.55
22:5n-6	0.05	0.04	0.01	n.s.	1.25
22:5n-3	0.15	0.10	0.02	n.s.	1.50
22:6n-3	0.15	0.18	0.02	<0.04	0.83

[a]From Bitman *et al.* (39). Normalcy ratio = value in disease/value in control. n.s., not significant.

Type I Hyperlipoproteinemia

Type I hyperlipoproteinemia (also known as Type I hyperlipidemia) (42) is characterized by the postprandial accumulation of chylomicrons in the circulation. This is a rare disease characterized by the absence of lipoprotein lipase activity. Since this enzyme regulates the tissue uptake of lipoprotein triglyceride, chylomicrons and very low density lipoprotein (VLDL), the carriers of plasma triglyceride accumulate in the circulation postprandially. The disorder can be primary (*i.e.*, absence of lipoprotein lipase) or secondary to (i) the absence of apoprotein C-II, the specific cofactor of lipoprotein lipase (43); or (ii) the presence of a lipoprotein lipase inhibitor (44). Two women with this disorder of lipoprotein metabolism have been studied during lactation (45–47). Table 10 provides data on the triglyceride fatty acid composition of the patients' milk taken at 3, 9 and 10 d *postpartum*. The milk fatty acid profile is compared to that of the mother's plasma and to the milk of a healthy control subject. Total milk triglycerides were much lower in the patient's milk than in control milk [1.5 and 0.9 g/dL at day 9 and 10, respectively, as compared to the normal value of 3.5–4.5 g/dL (10,11)]. The proportion of long-chain fatty acids in the patients' milk was much lower than that in healthy milk, suggesting that the absence of lipoprotein lipase activity also was evident in the lactating mammary gland. This conclusion is supported by the marked difference in long-chain fatty acids > 18:1 (*i.e.*, 18:1–20:4) between the patient's plasma and milk, levels being much higher in the circulation than in milk. It seems therefore, that long-chain fatty acids were not efficiently incorporated from circulating lipoprotein triglyceride into the lactating mammary gland, a process that is dependent upon lipoprotein lipase activity (14,15). Indeed, administration of heparin, which releases lipoprotein lipase from the endothelium into the circulation, failed to release measurable amounts of enzyme in this patient (45). Furthermore, the patient remained hypertriglyceridemic during lactation, indicating that circulating triglycerides were not taken up by the mammary gland for milk fat synthesis. To compensate for the low long-chain fatty acid levels, mammary gland synthesis of medium-chain fatty acids ($<C_{14}$) was markedly increased (Table 10). Structural analysis of the lipids showed that, as in healthy milk (10), the medium-chain fatty acids were present at the *sn*-3 position of the triglyceride molecule and were essentially absent in milk phosphatidylcholine.

TABLE 10

Triglyceride Fatty Acid Composition in the Milk of a Patient with Type I Hyperlipidemia Compared to That of Patient's Plasma and to That of Healthy Milk[a]

	Patient				Healthy milk	
		Milk				
Fatty acids	Plasma	Day 3	Day 9	Day 10	Day 2	Day 7
10:0	nd	nd	3.2	4.1	0.6	1.6
12:0	nd	5.8	14.9	21.3	3.0	6.4
14:0	3.2	8.9	21.6	29.9	5.3	7.0
16:0	31.6	27.8	26.0	21.8	26.5	23.0
16:1	6.6	2.7	1.5	0.7	4.0	3.7
18:0	5.9	5.7	5.9	4.7	7.8	5.7
18:1	33.6	39.6	20.9	13.5	37.6	31.3
18:2	13.5	7.6	4.1	2.8	10.0	16.6
20:1	2.0	0.8	0.8	0.8	0.6	0.7
20:2	0.8	0.6	0.4	nd	0.5	1.0
20:3	nd	nd	0.2	nd	0.4	0.8
20:4	1.0	0.2	nd	nd	0.8	0.6

[a]Data given as moles %; nd, not detectable. From Steiner *et al.* (45).

The composition of the patient's milk was closer to normal during the colostral phase (at day 3 *postpartum*), *i.e.*, higher amount of long-chain and proportionally lower level of medium-chain fatty acids. This raises the question whether in early lactation [a period when intercellular rather than transcellular transport from the blood into milk is evident in the mammary gland (7)] circulating lipoprotein triglycerides could enter the mammary gland by a lipoprotein lipase-independent mechanism. Low total fat, but normal levels of long-chain fatty acids are present in *prepartum* mammary secretion (48), a period when, contrary to *postpartum* milk, lipoprotein lipase activity cannot be detected in this secretion (49). Although the authors did not comment on other abnormalities of the milk of their patient (45), three other deviations from normal milk (50,51) were apparent—total cholesterol, phospholipid and FFA levels were all elevated. Whether these are due to milk collection, handling and analysis techniques or are associated specifically with Type I hyperlipidemia is not known. The milk of the second patient studied (47) had similar changes in fatty acid composition as the patient whose milk fatty acid analysis is presented in Table 10.

TABLE 11

Milk Lipids in Abetalipoproteinemia[a]

	Patient's milk			
Fatty acids	0.5 mo	3 mo	6 mo	Healthy milk
12:0	5.1	5.0	4.1	1.4 ± 3
14:0	22.0	37.2	31.2	3.8 ± 0.5
16:0	25.3	31.8	28.1	19.8 ± 0.9
16:1n-7	4.2	1.9	2.7	2.5 ± 0.3
18:0	3.7	2.7	3.4	8.3 ± 0.5
18:1n-9	33.0	15.6	23.9	38.2 ± 2.0
18:2n-6	1.6	2.7	3.0	18.8 ± 1.6
18:3n-3	0.1	0.1	0.1	0.3 ± 0.1
Total saturated	56.8	77.1	67.2	34.7 ± 2.4
Total monounsaturated	39.3	18.4	27.4	42.1 ± 0.5
Total n-6	2.1	3.5	4.2	20.1 ± 3.0
Total n-3	0.4	0.4	0.4	2.1 ± 0.5

[a]Fatty acid composition of breast milk from a patient with homozygous hypobetalipoproteinemia and normolipidemic lactating women. Data are expressed as mean ± SEM. From Wang and Illingworth (54).

Hypobetalipoproteinemia

In this rare disorder of lipid metabolism, apoprotein B is absent (52). As a consequence, chylomicron and VLDL, the major vehicles for the transport of triglyceride in the circulation, are absent from the plasma of these patients. Since chylomicron and VLDL-triglyceride are the main source of milk triglyceride fatty acids, one would expect a lower milk fat content, as well as a much lower concentration of long-chain fatty acids in the milk of these patients. One such patient studied first in the early 1980s (53) was followed after the delivery of her second child, when her milk fat composition was examined (54). The data presented in Table 11 show a milk fatty acid profile similar to Type I hyperlipidemia (Table 10) which is characterized by markedly lower levels of long-chain fatty acids and proportionally higher levels of medium-chain fatty acids (*i.e.*, 12:0–16:0). However, contrary to type I hyperlipidemia, there was also a shift in plasma fatty acid composition toward shorter chain components. Because patients with hypo- or abetalipoproteinemia are unable to synthesize chylomicrons or VLDL, their plasma triglyceride levels are consistently very low (about 10 mg/dL). The essential fatty acid deficiency was more pronounced in the milk of the hypobetalipoproteinemia patient (Table 11). Because of the important role of long-chain polyunsaturated fatty acids for brain development, prostaglandin synthesis and infant growth, breastfed infants of these patients might have to be supplemented with these fatty acids. While total milk fat content was lower than normal (Table 11, and refs. 10 and 11), milk protein content during the first six months of lactation was higher. It is interesting that milk lipoprotein lipase activity was higher in hypobetalipoproteinemia than in the milk of healthy women (54). This might represent a compensatory increase in enzyme activity in the lactating mammary gland with the aim to maximize the uptake of circulating long-chain triglyceride fatty acids.

Thus, two disorders of lipid metabolism that are char-

acterized either by the absence of the enzyme that regulates tissue uptake of circulating lipoprotein triglyceride or by the absence of triglyceride-rich lipoproteins from the circulation result in similar changes in milk fat amount and composition.

Atopic Eczema

A relationship between atopic eczema and abnormal serum essential fatty acids was first reported by Hansen in 1937 (55). Diets rich in polyunsaturated fatty acids were subsequently found to ameliorate the condition (56). More recent studies show that higher concentrations of linoleic acid are found in plasma phospholipids (57–59), leukocyte membrane lipids (60) and adipose tissue (61 cited in ref. 64) and proportionally lower levels of γ-linoleic and arachidonic acids in these subjects as compared to healthy individuals. Furthermore, these fatty acid abnormalities are present in umbilical cord sera, suggesting that they may have a role in the pathogenesis of atopic disease (62). These changes are consistent with lower than normal activity of Δ6 desaturase and a defect in the conversion of dietary linoleic acid to its long-chain polyunsaturated metabolites (63). Clinical improvement is achieved following treatment with evening primrose oil which is rich in linoleic acid. Recent studies show that the same abnormality in Δ6 desaturase activity is evident in the fatty acid composition of the milk of mothers of children with atopic eczema (64). The recent study of Wright and Bolton of Zimbabwean women (64) (Table 12) shows that whereas linoleic and α-linolenic acid levels were significantly higher in milk of mothers of atopic children, their metabolites, γ-linolenic, dihomo-γ-linolenic and arachidonic acids were all lower than in the milk of control women (64).

The difference in the essential fatty acids and their metabolites in the milk of mothers of healthy children as compared to those of children with atopic eczema suggests that the milk of the mothers of atopic children might not be able to alleviate the infants' symptoms. Indeed, milk of healthy women might delay the onset of symptoms in atopic infants (64). These findings explain the controversy about the role of breast feeding in the prevention or treatment at atopic eczema (65).

TABLE 12

Fatty Acid Composition of Total Breast Milk Lipids of Zimbabwean Mothers of Children with Atopic Eczema Compared with Controls[a]

	Mothers of atopic children (n = 25)		Control (n = 22)	
Fatty acids	Mean	SD	Mean	SD
14:0	5.5	1.7	5.4	1.8
16:0	22.4	4.3	22.9	5.8
18:0	8.4	2.8	7.9	2.6
Total saturated	36.6	—	36.2	—
16:1	2.9	0.8	3.1	1.2
18:1	27.0	7.1	25.2	6.4
Total monounsaturated	29.9	—	28.3	—
18:2n-6	12.8	3.6	10.2	3.4
18:3n-6	0.5	0.2	0.6	0.4
20:3n-6	0.5	0.3	0.7	0.4
20:4n-6	0.8	0.3	1.0	0.5
Total n-6	14.6	—	12.5	—
18:3n-3	0.8	0.3	0.6	0.4
20:5n-3	0.3	0.3	0.4	0.2
22:5n-3	0.5	0.3	0.6	0.3
Total n-3	1.6	—	1.6	—

[a]From Wright and Bolton (64).

TABLE 13

Fatty Acid Composition of Breast Milk of Mothers of Healthy Infants and of Infants with Neonatal Jaundice (wt%)[a]

Fatty acids	Jaundice	Healthy	Normalcy ratio jaundice/normal
6:0	0.01	0.02	0.50
8:0	0.16	0.14	1.14
10:0	1.54	1.26	1.22
10:0n-1	0.01	0.01	1.00
11:0	0.01	0.01	1.00
12:0	6.31	6.13	1.03
12:1n-1	0.02	0.02	1.00
13:0	0.01	0.01	1.00
14:0*br*	0.02	0.01	2.00
14:0	6.28	7.16	0.88
14:1n-5	0.24	0.19	1.26
15:0*br*	0.07	0.08	0.88
15:0	0.28	0.30	0.93
16:0*br*	0.12	0.07	1.71
16:0	20.64	20.76	0.99
16:1n-7	3.43	2.96	1.16
17:0*br*	0.17	0.16	1.06
17:0	0.37	0.34	1.09
17:1n-8	0.32	0.25	1.28
18:0*br*	0.02	0.01	2.00
18:0	7.08	6.47	1.09
18:1n-9	34.22	32.45	1.05
18:2n-6	14.25	15.58	0.91
18:3n-6	0.19	0.09	2.11
19:0	0.11	0.09	1.22
18:3n-3	1.02	1.06	0.96
18:4n-3	0.24	0.23	1.04
20:0	0.17	0.19	0.89
20:1n-9	0.54	0.70	0.77
20:2n-6	0.32	0.70	0.46
20:3n-6	0.36	0.50	0.72
20:4n-6	0.48	0.78	0.62
20:3n-3	0.03	0.07	0.43
20:4n-3	0.07	0.05	1.40
20:5n-3	0.11	0.03	3.67
22:0	0.10	0.11	0.91
22:1n-9	0.12	0.14	0.86
22:4n-6	0.07	0.26	0.27
22:6n-3	0.32	0.31	1.03
24:0	0.02	0.07	0.29
24:1n-9	0.03	0.10	0.30

[a]Authors' unpublished data; normalcy ratio = value in disease/value in control.

Breast Milk Jaundice

Within the first 3–4 d of life and later (within the 2nd and 3rd wk after birth), episodes of hyperbilirubinemia in the newborn have been associated with breastfeeding. Whereas the early postnatal jaundice is considered physiologic (66–68), the later onset of jaundice in breastfed infants is generally described as breast milk jaundice (66,69). High levels of free (unconjugated) bilirubin in these infants have led to the search for factors in milk that inhibit the conjugation of bilirubin. Initial studies suggested that the inhibitor might be an unusual steroid metabolite of progesterone, pregnane-3(α),20(β)-diol, that was shown to competitively inhibit the activity of hepatic glucuronyltransferase *in vitro* (70–72). However, later studies have failed to confirm this observation (73).

The high unconjugated bilirubin in infants with breast milk jaundice has led to the search for inhibitors of glucuronyltransferase in the abnormal milks. The finding that FFA inhibit the enzyme *in vitro* (74,75) resulted in studies that have quantitated the amount of FFA in jaundice-causing milks as compared with healthy milks (74–77). Furthermore, because FFA might be produced during the action of milk lipases (78–79) on the triglycerides of milk (11), several studies have examined this topic.

Although high activity of milk lipoprotein lipase (80), bile salt stimulated lipase (81,82) or "nonstimulated" lipase (83) could be associated with breast milk jaundice, this link has not been found (84–87). Furthermore, FFA levels also were found to be similar in healthy and in jaundice-causing milk (84,88,89). The association between FFA and increased unconjugated bilirubin level (either because of inhibition of the conjugation or because of increased absorption of bilirubin from the intestine) has led to studies of the amount and composition of the fat in jaundice-causing milk. Reports of significantly higher fat concentration in jaundice-causing than in healthy milk (90) have not been confirmed (Bitman, J., Hamosh, M., Gartner, L.M., and Wood, D.L., unpublished observations). Indeed, in the former study, fat was quantitated by the creamatocrit technique, a method that often overestimates milk fat, whereas in the latter study milk fat was quantitated by wide bore gas chromatography. However, while finding similar levels of total fat, Bitman and coworkers found differences in the concentration of specific fatty acids.

The fatty acid composition of the milk (7 specimens from 3 mothers of healthy infants and 10 specimens from 3 mothers of jaundiced infants) is presented in Tables 13 and 14. Differences in most fatty acids were small and the gross composition was similar in milks from the two groups for the major fatty acids, 10:0, 12:0, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3n-3, which comprise over 90% of milk fat. This can be seen readily in the normalcy ratios which compare the value for the diseased population to the comparable normal value. Normalcy ratios for these major fatty acids were close to 1.0, indicating little alteration in these fatty acids. However, although the gross composition of the major fatty acids was similar, a number of alterations were observed in the polyunsaturated fatty acids (PUFA) (Table 13 and 14) suggestive of metabolic aberrations. A 2% decrease in total PUFA was observed which was compensated for by an increase in monoenoic fatty acids (Table 14). Although the content of linoleic acid was only reduced by about 10%, there was a much greater decrease in the n-6 fatty acids derived from linoleate (Table 14). Thus, arachidonic acid, 20:4n-6, the principal PUFA metabolite, was only 62% of the normal amount (Table 13).

Desaturation and elongation reactions are involved in the step-wise metabolism of linoleic acid to fatty acids of 20-carbon atoms and longer. The normalcy ratios for desaturase enzymes indicated that Δ5 desaturase activity was reduced and conversely, Δ6 desaturation products were increased (Table 14). The products of elongation also were greatly reduced.

Recently, Uhari *et al.* (91) studied the role of fatty acids in neonatal jaundice. Immediately after delivery, a change was instituted in the diet of a group of mothers being fed the ordinary hospital diet, with a polyunsaturated diet [polyunsaturated to saturated ratio (P/S) = 1.5]. Although the breast milk readily reflected these dietary differences and showed high 18:2 levels, there were no differences in the incidence of neonatal jaundice in the infants of the two groups of mothers. Nine of the 145 newborn infants (6.2%) of mothers on the low P/S diet had jaundice as compared to 12 out of 187 (6.4%) of newborn infants of mothers on the high P/S diet. These dietary changes and changes in the breast milk compositions also were reflected in changes in the blood fatty acid patterns of the newborns ingesting these very dissimilar milks. Uhari *et al.* (91) concluded that the changes in the composition of the serum fatty acids had no effect on neontal jaundice.

In the present study, changes in patterns of the fatty acids in the milk from mothers of infants with jaundice were consistent with changes in polyunsaturated fatty acid metbolism. Similar changes in PUFA metabolism have been associated with a number of disease states characteristic of essential fatty acid deficiency (23,30,31).

The studies described in this review show that maternal

TABLE 14

Fatty Acid Patterns of Breast Milk Lipids of Mothers of Healthy and Jaundiced Infants (wt%)[a]

Fatty acids	Jaundice	Healthy	Normalcy ratio
Total polyunsaturated fatty acids	17.58	19.76	0.89
18:2n-6 + 20:4n-6	14.73	16.36	0.90
Total n-6 acids	16.67	17.91	0.87
Total n-6 18:2	1.42	2.33	0.61
Total n-3 acids	1.91	1.85	1.03
Total n-3 18:3	0.89	0.79	1.13
Total n-9 acids	34.91	33.39	1.05
Total n-9 18:1	0.69	0.94	0.73
Total monoenoic	38.92	36.81	1.06
Total saturated	43.09	43.06	1.00
20:4n-6/18:2n-6	0.03	0.05	0.60
22:4n-6/20:4n-6	0.15	0.33	0.40
Δ4 Desaturation products	0.32	0.31	1.03
Δ5 Desaturation products	0.59	0.81	0.73
Δ6 Desaturation products	0.19	0.09	2.11
Δ9 Desaturation products	37.89	35.60	1.06
Elongation products	0.94	1.61	0.58

[a]Authors' unpublished data; normalcy ratio = value in disease/value in control.

diseases result in changes in fatty acid composition and/or changes in lipid class composition of milk. However, in spite of these changes, the amount and composition of milk fat is compatible with normal infant development. Detailed dietary information was not provided in many of these studies; therefore, the possible effect of diet on the fatty acid composition of milk could not be assessed.

More studies are needed to assess the effect of maternal diseases that affect lipid metabolism on the composition of milk fat. Studies also are needed to evaluate whether such changes have long or short term effects on infant development.

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The Absorption of Fish Oils and Concentrates[1]

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Both preventive and curative therapies have created a considerable demand for eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. The most common sources for ω3 fatty acids are fish oil. The concentrations of EPA and DHA in commercial oils, after modest enrichment, reach about 300 mg/g; alternative technologies can produce reasonably priced fish oils containing 400 or even 500 mg/g of ω3 acids. When the acids are liberated from the glycerides, concentrates of ethyl esters or free acids with 65 to 70% total ω3 fatty acids (at least 50% EPA + DHA) are readily prepared. Difficulties have arisen because most clinical trials have used fish oils of unspecified composition, and some trials are now based on either ethyl esters or free acids. There are at least three different, but not mutually exclusive, absorption routes in humans, namely the preduodenal route, the lymphatic route *via* chylomicrons, and the route *via* the portal vein to the liver. This makes it difficult to compare results. The difficulty in obtaining dose-related clinical data may in part be due to the form in which the ω3 acids are offered and due in part to the natural presence of these fatty acids in the body. The nontriglyceride forms, especially the free acids, have been advocated for standardization of trials to facilitate interlaboratory comparisons.
Lipids 27, 858-862 (1992).

The epidemiology of the health benefits of ω3 fatty acids in Greenland Eskimos (1-5) has been the source of much discussion. Excluding difficulties in diagnosis and *post mortem* evaluations (5), the effect of dietary ω3 fatty acids could be attributed to an extremely high intake of long-chain ω3 fatty acids and to a possible role of ω3 docosapentaenoic acid (DPA).

It is well to remember that the studies on Greenland Eskimos were started nearly a decade before the first publication (6) on the potential role of ω3 fatty acids appeared in 1979. At the same time, a survey of Alaskan Eskimo consumption of fats and fatty acids was the subject of a U.S. doctoral thesis in 1973 and provided a comparable data base (7). Table 1 provides the proportions of the three important long-chain fatty acids in fats of several marine mammals from Alaska, the Arctic and the Atlantic coast of Canada. Other evidence has been published on fats of northern food sources (7,9,11), but fish fats basically have relatively low levels of DPA (12) compared to fats of seals and whales.

DPA and marine mammal fats. The function of DPA in marine mammal fat has been obscure, and it is not known to be linked to an eicosanoid in the same way as is eicosapentaenoic acid (EPA). In fact it seems rational to propose that

TABLE 1

Proportions (wt/wt% of total fatty acids) of Three Long-Chain ω3 Fatty Acids in Blubber Fats of Alaskan Marine Mammals, and of Atlantic Harbor Seal, Ringed Seal and Finwhale

Marine mammals	Fatty acid[a]			
	EPA	DPA	DHA	Reference
Walrus (Pacific) *(Odobenus rosmarus)*	7.63	5.99	5.83	(7)
Bearded seal (Pacific) *(Erignathus barbatus)*	8.59	5.99	6.73	(7)
Pacific harbor seal *(Phoca vitulina richardi)*	8.67	4.92	8.33	(7)
Pacific bowhead whale *(Balaena mysticetus)*	8.52	3.23	5.22	(7)
Atlantic harbor seal *(Phoca vitulina)*	4.35	3.95	8.09	(8)
Atlantic finwhale *(Balaenoptera physalus)*	3.72	2.28	6.23	(10)
Ringed seal *(Phoca hispida)*	9.8	5.9	10.0	(9)

[a]EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid, DPA, docosapentaenoic acid.

in most fatty tissue the Δ4,5 desaturase postulated to convert DPA to docosahexaenoic acid (DHA) is relatively inactive. Brain and neural tissues are clearly exceptions, but in humans relatively little work has been done on EPA-DPA-DHA proportions in whole body studies (13) as compared to that on the blood platelet phospholipids (14) which may, however, not be representative. Instead it has become increasingly apparent that DPA is a temporary storage site for surplus EPA. The hypothesis that DHA has no specific role in movine mammal fats could also account for the accumulation of DPA in the seal fats (Table 1). In the fish eaten by seals, the DPA level is usually only 2-5% of combined EPA and DHA (12).

Triglycerides. Long-chain ω3 fatty acids, especially DHA, are reputedly located in the *sn*-3 position of marine mammal triglycerides, as distinct from the fish oils where DHA is found mainly in the *sn*-2 position, (15-18). H. Brockerhoff (cited in ref. 16) published much of the data and theory on this difference.

The fats in the foods eaten by humans have been dealt with by various authors (19,20). The major fat types are triglycerides, followed in nutritional importance by phospholipid or other "polar" lipid and finally by free fatty acids. Ethyl esters will also be included here.

In small populations consuming a high level of marine fats, pros and cons exist as to the benefits of a high ω3 fatty acid intake (21-23). In other groups (24,25), it is difficult to demonstrate health benefits of eating fish (26) on a par with those of the Kromhout study (27). The latter study was supported by retrospective examination of fish consumption in other diet-health studies (28,29). In the recent spectacular extension of survival in patients advised to eat more fatty fish three times a week (30), the most interesting result was that the benefits became apparent in just over three months. In the study by Burr *et al.* (30), fish oil cap-

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Abbreviations: DHA, 4,7,10,13,16,19-docosahexaenoic acid, 22:6ω3; DPA, 7,10,13,16,19-docosapentaenoic acid, 22:5ω3; EPA, 5,8,11,14,17-eicosapentaenoic acid, 20:5ω3; FFA, free fatty acid(s); HDL, high density lipoprotein; MG, monoglyceride(s); PUFA, polyunsaturated fatty acid(s); TG, triglyceride(s).

sules were provided as an alternative for those patients not wishing to consume large quantities of fish.

Even fish oil studies of short duration can be relevant. For example, Boyce and Fordyce (31) fed cod liver oil for 14 d as either a bolus of oil while fasting or with lunch. The subjects then had a washout period of 14 d and then reversed the consumption pattern. The ratio of high density lipoprotein (HDL) cholesterol to total cholesterol changed according to the time of consumption. A favorable result, an increase in HDL levels, followed from taking capsules with meals.

Free acids in the diet are totally absorbed, and fatty acids of triglycerides are usually absorbed to the extent of 90% or more (32,33). The distribution of long-chain ω3 fatty acids on the glycerol moiety differs between fish and seals or other marine mammals (15), including seal milks (34) but is of course limited to a total of about 30 mole% for all ω3 fatty acids in all natural triglyceride (17). Thus U.S. menhaden oil (see Table 2) might have (in wt%) 9.4% DHA, 2.1% DPA, 14.8% EPA and 3.3% 18:4n-3 (35). By comparison the ubiquitous MaxEPA (Seven Seas Health Care, Hull, U.K.) has a nominal label composition (wt %) of 18% EPA and 12% DHA which, in parallel with other "oils," may not be the actually correct figure in some samples (36). In one product, a "reesterified" ω3 polyunsaturated fatty acid (PUFA)-enriched triglyceride (37–39), the concentrations were: EPA 34%; DPA 3.5%; DHA 19.0% (wt/wt% basis). However the distribution of fatty acids on glycerol in such highly enriched triglyceride oils may not follow that expected for natural fish oils. It is widely accepted based on the work of Brockerhoff and others (cited in 16–18) that in fish oils EPA should be primarily in the 2-position, and DHA should be distributed according to a formula: *sn*-1, 0.28x; *sn*-2, 2.06x; *sn*-3, 0.66x, where x = the mole% total of DHA. Table 2 shows a recent analysis of an encapsulated product in which more EPA is found in positions 1 and 3 than in position 2, and the DHA is higher in position 3 than in position 2. For comparison menhaden oil data are included to confirm that DHA should be located primarily in the 2-position. EPA is less specific in distribution. A recent investigation on the hydrolysis of menhaden oil (40) showed that pancreatic lipase would hydrolyze fatty acids of the outer (1 and 3) positions of triglycerides at different rates related to ethylenic bond positions nearest the carboxyl group, but independently of chain length and number of double bonds (Table 3). The fatty acids liberated from glycerol may follow different distribution routes in the body (Fig. 1) and are not necessarily reassembled with the 2-monoglyceride in chylomicrons.

Ethyl esters and free acids. An increasing number of biomedical papers have reported the use of ethyl esters (42–48). These can offer a high concentration of ω3 fatty acids based on urea complexing (35), or urea complexing combined with supercritical CO_2 (49) or chromatographic techniques (50). The hydrolysis of ethyl esters by pancreatic lipase *in vitro* is 10–50 times slower than that of triglycerides (40). This is not the same as *in vivo* hydrolysis by this lipase in the intestinal lumen (41). The possibility of hydrolysis in the intestinal wall after absorption, as shown in Figure 1, is suggested by the preferential incorporation of EPA, administered as ethyl esters, into plasma phospholipids (51). There is a basic difference in the effects of EPA, as acids or esters, on arachidonic acid in human plasma (52).

The normal human diet does not contain much fatty acid in free form. There is, however, considerable lipolytic activity present in humans which affects esters prior to the important intestinal lipolytic activity; lipase is the major enzyme found in human gastric juice (53,54). In piglet stomachs, fish oil was broken down to the extent of 50% to diglyceride and free fatty acids (55). Iverson (56) has shown that there is rapid gastric hydrolysis of seal milk triglycerides in the stomach of nursing grey seal pups, and Puppione *et al.* (34) have suggested more studies on this form of fat absorption and transport. In humans, a feedback mechanism may limit hydrolysis (57), but local absorption of EPA or DHA is still possible. One reference to a beneficial effect of fish oil in the stomach (58) may be pertinent.

There are numerous problems created by the various forms of administration possible for the long-chain ω3 fatty acids in humans. The enriched triglycerides may have abnormal fatty acid distributions on the glycerol backbone and this information is rarely provided. Presumably enzymes are used to add an enriched fatty acid mixture to glycerol (59). Usually the total for EPA and DHA is indicative of one or more such fatty acids per molecule, but as Figure 1 indicates, whether these go to the liver or are circulated as chylomicron triglycerides is an open question. That ethyl esters are fully absorbed is now known (60). It is reported that 6 g of ethyl ester per day is totally absorbed (Horrobin,

TABLE 2

Mole Percent Distribution of Selected Fatty Acids on Glycerol of Efamol Fish Oil[a,b] and of Menhaden Oil[c]

Glycerol position	Fatty acid			
	18:4ω3	20:5ω3	22:5ω3	22:6ω3
Efamol total	4.0	18.0	2.2	10.8
sn-1% in position	4.0	15.0	1.0	2.0
% of total	(33.0)	(27.0)	(11.0)	(5.0)
sn-2% in position	5.0	18.0	4.0	15.0
% of total	(42.0)	(33.0)	(63.0)	(42.0)
sn-3% in position	3.0	21.0	2.0	20.0
% of total	(26.0)	(39.0)	(26.0)	(53.0)
Menhaden total	3.6	16.9	2.3	9.1
sn-1 and *sn*-3	3.6	13.2	1.2	6.6
sn-2	3.7	11.7	2.8	9.8

[a]Adapted from Lawson and Hughes (33).
[b]MaxEPA is stated (33) to be similar.
[c]Adapted from Yang *et al.* (40).

TABLE 3

Percentage of Selected Fatty Acids (mole percent) in Free Fatty Acids and Monoacylglycerols of Rat Lumenal Lipids During Lipolysis of Menhaden Oil Triacylglycerols[a] and from *in vitro* (20%) Lipolysis[b]

Lipid class	Fatty acid			
	18:4ω3	20:5ω3	22:5ω3	22:6ω3
Total in TG[c]	3.6	16.9	2.3	9.1
FFA[d] in lumen	3.6	13.9	1.6	4.8
MG[e] in lumen	3.5	14.6	3.8	18.0
FFA (*in vitro*)	1.4	4.1	0.8	1.7
MG (*in vitro*)	4.4	13.1	3.0	10.8

[a]Adapted from Yang *et al.* (41).
[b]Adapted from Yang *et al.* (40).
[c]TG, triglycerides.
[d]FFA, free fatty acid.
[e]MG, monoglyceride.

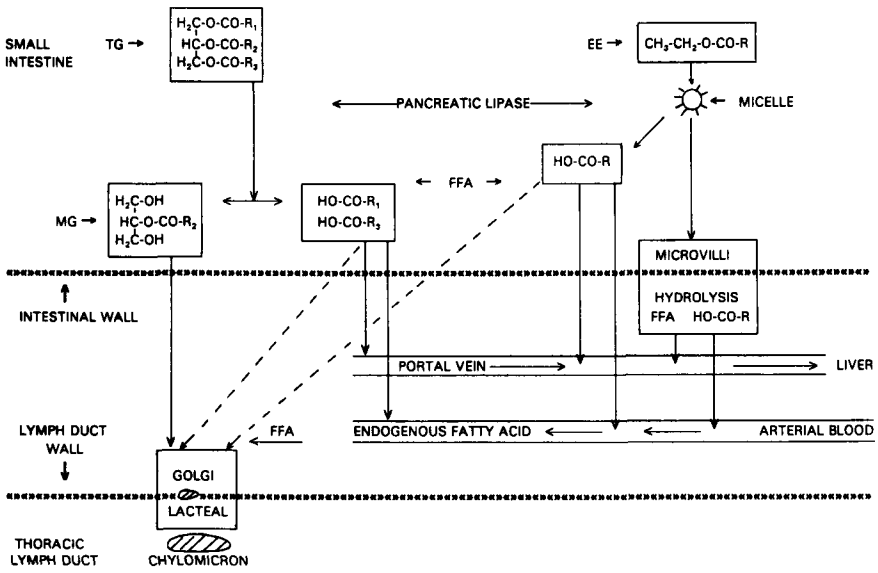

FIG. 1. Some possible origins for fatty acids found in chylomicrons, with special reference to hydrolysis of ethyl esters. The action of pancreatic lipase on ethyl esters *in vivo* and *in vitro* is described elsewhere in detail (40,41).

private communication), and as much as 15 g/day has been administered. Another report on blood pressure suggests that 3 g/day is effective but 6 g/day does not double the clinical effect (45). No ethyl esters have been reported in the blood lipids of humans given highly purified EPA in that form (61), so it is evidently totally hydrolyzed during digestion. Since 60–70% (by weight) concentrates of EPA plus DPA plus DHA are readily prepared, the 6 g/day yields about 4 g/day of the ω3 fatty acids, which, depending on the symptoms, could be a therapeutic dosage (45,62–64). As little as 300 mg/day could be a "preventive" (in cardiovascular terms) dosage (64). Ethyl esters, which are already established encapsulated products in the health supplement market, could in most cases be free from cholesterol and saturated acids (Fig. 2). The possibility of comparing different clinical studies on a dose basis is also clear. This is, at present, often virtually impossible with triglycerides for the reasons given above, but the nonlinear absorption of ethyl esters creates another problem.

Free acids have been reputed to irritate the stomach. This may refer in practice to shorter chain fatty acids, for example butyric acid from rancid butter. Historically, very thorough mastication of foods was practiced at various times, usually with beneficial results (65). Lingual gastric lipase could have been effective, in such cases, in providing all types of free acids for gastric absorption (66). Another report refers to avoiding esophageal irritation through the use of free acids in capsules (33). Since most encapsulated ω3 products are taken with food, there does not seem then to be a serious objection to their use in free acid form beyond some eructation (48). Co-absorption with other fats is well established, and if gastric absorption is rapid, it may explain why the plasma triglyceride input from free acid sometimes follows the triglyceride rise (67) or precedes it (33). Gastric lipases actually produce free acids *in vitro* (54,57), a further indication that free acids are probably well accepted as reported elsewhere (48).

Ethyl esters of ω3 fatty acids could be used for encapsulated supplements or enteral products, but triglycerides are traditional for parenteral nutrition. A new challenge is then to consider whether highly concentrated free acids could be administered in this way, perhaps as salts (68) or absorbed on albumin, or even simply dissolved in the triglycerides already in these products (69). The point is that relative to the total fat only 1 or 2% of long-chain ω3 fatty

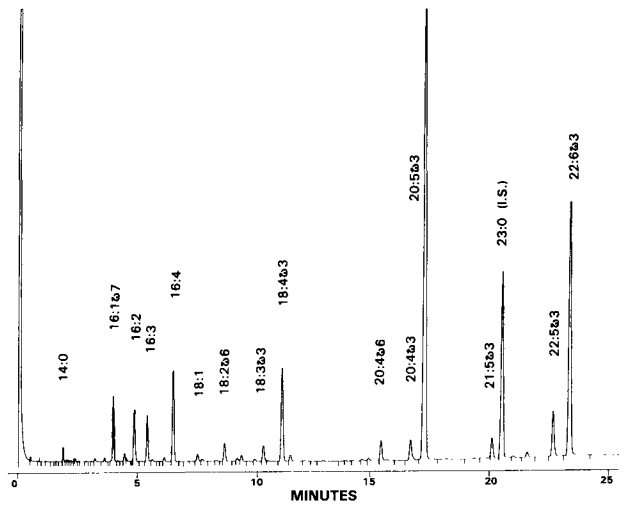

FIG. 2. Gas-liquid chromatographic analysis of a retail fish oil concentrate sold in ethyl ester form, with added ethyl 23:0 internal standard for quantitation. A bonded polyglycol Omegawax-320 flexible fused silica column (Supelco Inc., Bellefonte, PA) (30 × 0.32 mm i.d.) was operated in a Perkin-Elmer model 8420 gas chromatograph (GC, Norwalk, CT). GC oven temperature: 195° for 8 min, program at 3°/min to 240°, hold at 240°.

acids (EPA, DPA, DHA) needs to be supplied if it is in the form of a 60–70% free fatty acid concentrate. Like the ethyl esters, free acid concentrates can be obtained free of saturated acids, cholesterol, etc. Whether the free acids so administered are in any way toxic or are more immediately beneficial compared to triglycerides are some of the questtions to be answered.

Fish. Fatty fish eaten in moderation (30,70), or as a steady diet (71,72), show no obvious side effects. However, fish can be expensive and is not always a popular food in many population groups (73). It is surely important to provide access to fish oil fatty acids for those who do not or cannot eat fish, despite numerous warnings of risks (74), most of which are somewhat hypothetical compared to the real risks and side effects often associated with potent synthetic drugs. In contrast, the long-chain ω3 fatty acids, whether from fish or fish oil concentrates of any type, are purely natural dietary components and part of a system of checks and balances normal in the human body (75,76).

For example fish oil administration to achieve triglyceride reduction (77) may be accompanied by an increase in plasma glucose, which can be offset by increased administration of vitamin E (78). The latest considered opinions of experts (79,80) are that we are still grappling with various aspects of functions of ω3 fatty acids in what are instances of adult-onset degenerative diseases, a rather different problem from that of treating acute disease states. Peculiarly, our understanding of nutritional aspects of longer-chain ω3 fatty acids for early human development is not much further advanced at the other end of our life spans (81,82).

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ARTICLE

Effect of Fish Oil on the Fatty Acid Composition of Human Milk and Maternal and Infant Erythrocytes[1]

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To examine the effect of fish oil supplementation on the fatty acid (FA) composition of human milk and maternal and infant erythrocytes, five lactating women were supplemented with 6 g of fish oil daily for 21 d. Usual maternal diets contained 1,147 mg of total n-3 FA, with 120 mg from very long-chain (>C_{18}) n-3 FA. Supplementation increased dietary levels to 3,092 mg of total n-3 FA and 2,006 mg of very long-chain n-3 FA. Milk samples were collected daily, prior to fish oil ingestion, and at 4-h intervals on days 1, 7, 14 and 21. Milk n-3 FA content increased within 8 h and reached steady state levels within one week. The n-6 fatty acid content decreased. Erythrocyte eicosapentaenoic acid content increased from 0.24% to 1.4% ($P < 0.01$) in mothers and from 0.11% to 0.70% ($P < 0.05$) in infants. Docosapentaenoic acid increased from 1.4% to 2.2% ($P < 0.05$) in mothers and from 0.30% to 0.78% ($P < 0.01$) in infants. There was no significant change in docosahexaenoic acid or n-6 fatty acid content. Maternal platelet aggregation responses were variable. No differences in milk or plasma tocopherol levels were noted.
Lipids 27, 863-869 (1992).

Animals are unable to synthesize n-6 and n-3 fatty acids and are dependent on dietary sources. Linoleic (18:2n-6) and linolenic (18:3n-3) acids can be converted into their respective families of very long-chain polyunsaturated fatty acids (VLC-PUFA). However, because both fatty acid classes are elongated and desaturated *via* the same enzyme system, the presence of high levels of 18:2n-6 relative to 18:3n-3 may limit the formation of n-3 VLC-PUFA (1,2). The acids of interest derived from 18:3n-3 are eicosapentaenoic (EPA, 20:5n-3), docosapentaenoic (DPA, 22:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids.

Supplementation with 18:3n-3 has been shown to be less effective than fish oil for increasing tissue n-3 VLC-PUFA content (3), and preferential incorporation of dietary VLC-PUFA over their precursors into rat liver and brain has been demonstrated (4). These findings raise the possibility that preformed n-3 VLC-PUFA from the diet are an important source of n-3 VLC-PUFA for tissues.

There is increasing evidence that the n-3 VLC-PUFA have an important role in the structure and function of biological membranes, particularly in brain and retinal tissues. In animals, retinal depletion of DHA has been shown to result in altered electroretinogram response (5-7) and decreased visual acuity (8). Decreased learning ability of rats fed 18:3n-3 deficient diets has been demonstrated, corresponding with the brain DHA depletion (9,10). Visual and neurologic changes attributed to n-3 PUFA deficiency have been reported (11,12) in humans, but it is difficult to separate n-3 PUFA deficiency from other nutrient deficiencies in these studies.

Ascertaining the effect of diet on the fatty acid composition of brain and retinal tissues in humans must be accomplished using indirect markers such as erythrocytes and plasma. Based upon studies in rats and piglets which show that neural and erythrocyte tissues have similar relative effects from dietary modification of n-3 PUFA (13,14), it is likely that human erythrocytes are appropriate indicators of brain n-3 VLC-PUFA composition.

In addition to the potential role of DHA in tissue membranes, the n-3 VLC-PUFA EPA is a precursor for eicosanoids, which are potent metabolic effectors associated with inflammation and platelet aggregation (15,16).

The potential importance of the n-3 VLC-PUFA during perinatal growth and development has stimulated research into the effects of infant feeding practices on the fatty acid composition of infant tissues. Human milk contains small amounts of n-3 VLC-PUFA in addition to 18:3n-3, whereas commercial infant formulas contain only 18:3n-3. Differences between the plasma and erythrocyte fatty acid profiles of breast-fed and formula-fed infants have been observed (17-20). Fish oil supplementation of formula fed to preterm infants resulted in erythrocyte phospholipid DHA levels more similar to those of infants fed human milk (21). The requirement for n-3 VLC-PUFA for optimal infant health and development has not been determined, although recommendations have been made (22).

Maternal dietary fatty acid composition affects the fatty acid composition of breast milk with the PUFA content responding markedly (23-26). The n-3 VLC-PUFA content of human milk can be increased above the levels observed in women consuming a Western omnivorian diet. Inuit women consume high levels of dietary n-3 VLC-PUFA and had significantly higher levels of EPA and DHA in their milk compared to Vancouver controls (27). Harris *et al.* (28) supplemented the usual intake of breast-feeding mothers with fish oil, and dose dependent increases in the n-3 PUFA content of the milk were observed. The effect of high levels of n-3 fatty acids in human milk on infant tissues has not been determined.

A potential side effect of increased consumption of fish and fish oil is that it may increase the need for vitamin E.

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Abbreviations: ADP, adenosine phosphate; ANOVA, analysis of variance; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid(s); GLC, gas-liquid chromatography; HPLC, high-pressure liquid chromatography; P/S, polyunsaturated/saturated ratio; RDA, recommended daily allowance; VLC-PUFA, very long-chain polyunsaturated fatty acid(s) (longer than C_{18}).

In animals, fish oil supplementation has been shown to induce tissue vitamin E deficiency, even with excess dietary supply (29). Hartog *et al.* (30) found no adverse effects of fish oil supplementation on tissues at about twice the vitamin E dosage reported above. Vitamin E supplementation led to decreased susceptibility to *in vitro* peroxidation in tissues from rats fed diets supplemented with menhaden oil (31). It is not known whether humans are susceptible to disturbances in vitamin E metabolism due to increased n-3 PUFA consumption. However, in populations ingesting high levels of n-3 fatty acids, vitamin E deficiency is usually not seen (32).

The goal of the present research was to provide data on the changes in the fatty acid composition of breast milk and maternal and infant erythrocytes during maternal fish oil supplementation at a level equivalent to consuming a 4-oz portion of salmon daily. In addition, maternal dietary intake, milk and plasma vitamin E levels and maternal platelet aggregation response were measured. This information may help determine the optimum intake of n-3 fatty acids during infancy and maternal dietary requirements during lactation.

MATERIALS AND METHODS

Study design and data collection. Five lactating women and their infants were recruited for the study. All subjects signed an informed consent form, and the study had human subjects' approval from The University of Connecticut.

The supplementation period was 21 d, between 2 and 5 wk *postpartum.* None of the subjects were consuming any medications which might alter platelet aggregation. Infants received breast milk as their sole source of nutrient intake from birth and throughout the study. Mothers and infants were weighed weekly.

Mothers supplemented their usual diets with six 1-g capsules of Bio-EFA (PGE Technology, Cambridge, MA) daily. Capsules were consumed once daily at approximately the same time each day. Each capsule contained 180 mg EPA and 120 mg DHA, confirmed by gas-liquid chromatography (GLC), and 1 mg of vitamin E. This provided 1,080 mg EPA, 720 mg DHA and six mg of vitamin E per day.

Dietary intake was evaluated by collecting food records for six days (33,34), which were divided into three randomly assigned 48-h periods during the study. At least one weekend day was included. Forms with instructions to record all food and drink consumed and a 2-dimensional visual aid for estimating portion sizes of food and beverages were provided (cited in 35). Overall nutrient composition (Massachusetts Nutrient Data Bank, University of Massachusetts, Amherst, MA), α-tocopherol content (36), and the fatty acid composition of the diets were calculated (37).

Milk samples were collected immediately prior to ingestion of fish oil capsules each day. On days 1 (baseline), 7, 14 and 21, samples were also collected at 4, 8, 12 and 16 h postsupplementation. All samples were collected using an Egnell electric breast pump (Egnell Inc., Cary, IL). Mothers did not use any creams or oils on their breasts during the study. The pump was held to one breast until completely emptied, and an aliquot was taken for analysis. Samples were placed in the home freezer and stored for a maximum of 3 d, transported on dry ice, then stored at −70°C until analysis.

Blood was collected by a physician on days 1 and 21. Maternal blood was sampled by brachial arm venipuncture, and infant blood by heel prick.

Sample analysis. Total lipid was extracted from human milk using a modified Folch procedure (38). Total lipid weight was determined gravimetrically. Lipids were converted to methyl esters for analysis by GLC (39). Fatty acid methyl esters were dissolved in heptane to achieve a concentration of approximately 35 g/L for GLC analysis.

GLC analysis was performed on a Varian Vista 6000 gas chromatograph (Varian Associates, Sunnyvale, CA) using a Supelcowax 10 fused silica capillary column (30 m × 0.34 mm (Supelco Inc., Bellefonte, PA). Temperature programming was as follows: 180°C for 20 min, then increased 5°C/min to the final temperature of 200°C, and held for 20 min.

Quantification was based on relative peak areas as determined by a Hewlett-Packard 3390A integrator (Hewlett-Packard Co., Avondale, PA). Peak identification was made based on relative retention times of two external standard mixtures, PUFA1 and PUFA2 (Supelco).

Blood for the analysis of erythrocyte fatty acid composition was collected and prepared according to the method of Rose and Oklander (40), and fatty acids were methylated for GLC analysis as described above.

Blood for the measurement of platelet aggregation was collected separately into citrated vacutainer tubes. Platelet-rich plasma was obtained by centrifugation at 120 × *g* for 10 min. Platelet-poor plasma was obtained by centrifuging the remaining blood at 1,500 × *g* for 15 min. Platelet-rich and platelet-poor plasmas were prepared within one hour, and aggregation response was measured within 6 h of sample collection. Platelet aggregation response to 1, 2 and 4 μM concentrations of adenosine diphosphate (ADP) was measured in platelet-rich plasma using a Chronolog-Lumi Aggregometer (Chronolog, Philadelphia, PA).

Milk lipid for tocopherol analysis was extracted by the modified Folch prcoedure described above and dissolved in hexane for high-performance liquid chromatography (HPLC). Plasma lipids were extracted by the method of Driscoll *et al.* (41). Lipid weight was determined gravimetrically. Instrumentation for HPLC analysis and methods for quantification of vitamin E in both milk and plasma samples were as described by Lammi-Keefe (42).

Statistical analysis. Erythrocyte fatty acid composition, tocopherol levels in milk and plasma and platelet aggregation response were analyzed by a paired T-test (43). Components in milk samples taken at 4-h intervals during the day were analyzed by repeated measures analysis of variance (ANOVA) (44).

To determine if there was a plateauing, after an initial rise in the n-3 PUFA content of baseline milk samples, a two-step procedure in SAS was used (45). First, a spline model was fitted through the data to characterize the shape of the curve and to determine the approximate location of the knot, the point at which no further increase was observed. For data to the right of the knot, the hypothesis that there was no trend in the data was tested using repeated measures univariate ANOVA. The significance of the linear, quadratic and cubic components of the orthagonal polynomial contrasts generated by the SAS statement summary were analyzed.

RESULTS

Five mother-infant pairs participated in the study. All subjects who volunteered completed the study, and no deleterious side effects were reported. Infants were full term, between 37–42 wk gestation, and gained weight normally (mean 35 g per day) during the study. Maternal weight for all subjects remained constant within 1 kg.

Maternal diet analysis. Nutrient composition was analyzed for each of the 6 d of food record data collected, and an average intake calculated. The average intake of α-tocopherol for subjects during this study was 1.9 mg (range 0.8–5.1 mg). Subjects consumed an average of 34% of their calories as fat with a polyunsaturated to saturated fat (P/S) ratio of 0.4 and 376 mg cholesterol per day.

Average daily intake of n-3 fatty acids, the n-6/n-3 ratio and the change in dietary fatty acid composition when fish oil was supplemented are shown in Table 1. The study population consumed an average of 1,148 mg of dietary n-3 PUFA with 121 mg from the n-3 VLC-PUFA. Fish oil supplementation tripled dietary intake of n-3 PUFA to an average of 3,093 mg. Over 15 times more n-3 VLC-PUFA, 2,007 mg, was consumed. Total n-6/n-3 PUFA ratio decreased from 9 to 3.

Daily measurement of the fatty acid composition of human milk. The content of individual fatty acids in human milk before and after the study period is presented in Table 2. No significant changes in 18:2n-6 or 18:3n-3 were noted. Increases were: total n-3 VLC-PUFA 162%, EPA 525%, DPA 143% and DHA 89%. Total n-6 VLC-PUFA levels decreased 30%, and the ratio of n-6/n-3 VLC-PUFA decreased 275%. The ratio of total n-6/n-3 fatty acids decreased 45%. The P/S ratio of the milk was similar to that of the maternal diet and was not affected by the supplement.

Mean total milk lipid levels (±SD) for the 5 subjects were 2.74 ± 1.06 g/dL on day 1, and 2.10 ± 0.83 g/dL on day 21, not significantly different.

Analysis of the daily changes in fatty acid composition during the study are presented in Figure 1. There was a significant linear increase ($P = 0.001$) in total saturated fatty acid content and a quadratic decrease in total monounsaturated fatty acid content ($P = 0.02$) during the study (data not shown). The LC n-3 PUFA in human milk were best fit to a cubic-constant spline model. The equation for this model is as follows:

$$\text{Wt\%} = \text{BO} + \text{B1Z} + \text{B2U} + \text{E} \qquad [1]$$

where $z = [(\text{Day-6}) - (\text{Day-6})_+]$, $U = [(\text{Day-6})^2 - (\text{day-6})_+^2]$, $w = [(\text{Day-6})^3 - (\text{Day-6})_+^3]$.

Figure 1a–d shows the plot of EPA, DPA and DHA, and total n-3 VLC-PUFA contents using this model. For EPA, the knot was estimated to be at day 6.2, with correlation coefficient (r/2) of 0.38, and parameter estimates B/O = 0.4424, B/1 = 0.1357, B/2 = 0.0578 and B/3 = 0.0090. For DPA, the knot was estimated to be at day 6.6, with r/2 of 0.43 and parameter estimates B/O = 0.3463, B/1 = 0.3463, B/1 = 0.0711, B/2 = 0.0208 and B/3 = 0.0078. For DHA, the knot was estimated to be at day 6.3, with r/2 of 0.22, and parameter estimates B/O = 0.6604, B/1 = 0.1974, B/2 = 0.0851 and B/3 = 0.0115. For total n-3 VLC-PUFA (EPA + DPA + DHA) content, the knot was estimated to be at day 6.3, with r/2 of 0.35, and parameter estimates B/O = 1.446, B/1 = 0.4450, B/2 = 0.1858 and B/3 = 0.0263. Univariate analysis supported the hypothesis that there was no significant change to the right of the knot.

Figure 1E is the plot of total n-6 VLC-PUFA (20:2 + 20:3 + 20:4 + 22:4) content during the study. The decline in n-6 fatty acid content was best depicted using a cubic-linear spline model because levels continued to decrease significantly to the right of the knot ($P = 0.004$). Nonlinear estimation of the knot was day 6.5, with r/2 of 0.30. The equation for the cubic-linear model is:

$$\text{wt\%} = \text{B/O} + \text{B/1 (Day L)} + \text{B/2U} + \text{b/3W} + \text{E} \qquad [2]$$

TABLE 1

Dietary Intake of n-3 Fatty Acids[a]

	18:3n-3 (mg/d)	20:5n-3 (mg/d)	22:5n-3 (mg/d)	22:6n-3 (mg/d)	n-6/n-3 ratio (mg/d)
Mean ± SD	1027 ± 352	31 ± 42	7 ± 3	82 ± 106	9 ± 2
Range	760–1574	4–103	4–10	18–267	6–11
+FO	1086	1120	22	864	3

[a]Values are based on 6 d of food record analysis. +FO denotes increased intake with fish oil supplementation. SD, standard deviation.

TABLE 2

Fatty Acid Composition of Human Milk Before and After Supplementation with Fish Oil[a]

Fatty acids	Day 1	Day 21
12:0	4.8 ± 1.9	5.4 ± 2.0
14:0	5.3 ± 1.5	6.5 ± 1.6
16:0	20.6 ± 2.3	21.2 ± 3.0
16:1	2.9 ± 0.7	2.8 ± 0.7
18:0	6.5 ± 1.6	.8 ± 1.5
18:1	38.6 ± 3.1	35.0 ± 2.5
18:2n-6	13.0 ± 1.7	12.5 ± 3.0
18:3n-6	0.15 ± 0.07	0.15 ± 0.12
18:3n-3	0.77 ± 0.12	0.76 ± 0.23
20:1	0.67 ± 0.20	0.73 ± 0.11
20:2n-6	0.39 ± 0.12	0.23 ± 0.05
20:3n-6	0.55 ± 0.16	0.45 ± 0.26
20:4n-6	0.67 ± 0.11	0.52 ± 0.10
20:5n-3	0.08 ± 0.04	0.50 ± 0.12[b]
22:1	0.20 ± 0.06	0.18 ± 0.05
22:4n-6	0.24 ± 0.16	0.21 ± 0.15
22:5n-3	0.14 ± 0.05	0.34 ± 0.08[b]
22:6n-3	0.37 ± 0.26	0.70 ± 0.12[b]
Total polyunsaturated	16.5 ± 1.6	16.5 ± 3.2
Total saturated	37.2 ± 3.6	39.9 ± 3.8[b]
P/S ratio	0.45 ± 0.08	0.42 ± 0.12
Total n-6	15.0 ± 1.5	14.1 ± 2.9
Total n-3	1.4 ± 0.4	2.3 ± 0.5
n-6/n-3 ratio	11.0 ± 2.7	6.1 ± 1.0
Total monounsaturated	42.4 ± 2.9	38.6 ± 2.7[b]
Total n-6 VLC-PUFA	1.85 ± 0.34	1.42 ± 0.29[b]
Total n-3 VLC-PUFA	0.59 ± 0.35	1.55 ± 0.29[b]
n-3/n-3 VLC-PUFA ratio	3.60 ± 1.10	0.96 ± 0.30[b]

[a]Values are daily mean wt% ± SD (n = 5). P/S, polyunsaturated/saturated ratio. VLC-PUFA, very long-chain-polyunsaturated fatty acids.

[b]Values are significantly different by nonlinear regression analysis.

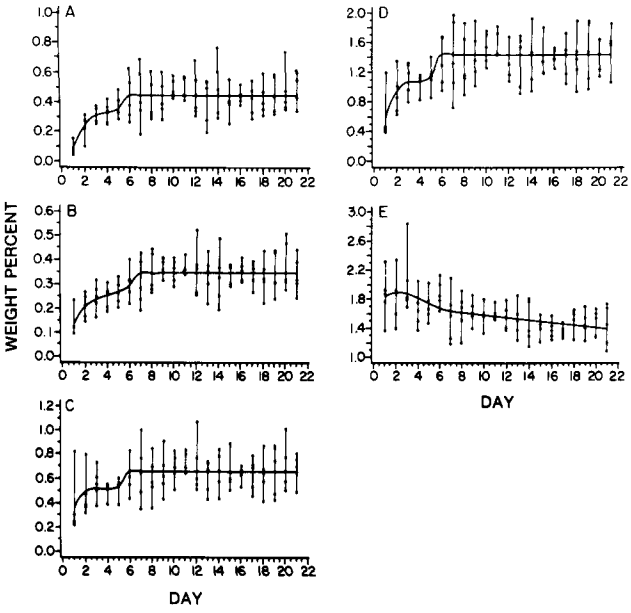

FIG. 1. Fatty acids in human milk during fish oil supplementation, plot of spline fit over raw data. A. Eicosapentaenoic acid (EPA), cubic-constant spline, assumed knot 6; B. Docosapentaenoic acid (DPA), cubic-constant spline, assumed knot 7; C. Docosahexaenoic acid (DHA), cubic-constant spline, assumed knot 6; D. Total n-3 very long-chain polyunsaturated fatty acid (VLC-PUFA), cubic-constant spline, assumed knot 6; E. Total n-6 VLC-PUFA, cubic-linear spline, assumed knot 7.

where Day L = (day-7), U = $[(\text{Day } 7)^2 - (\text{day-}7)_+^2]$, w = $[(\text{Day-}7)^3 - (\text{Day-}7)_+^3]$. Parameter estimates for this model are B/O = 1.635, B/1 = 0.0179, B/2 = 0.0260 and B/3 = 0.0039.

n-3 PUFA composition of milk sampled at four-hour intervals. Figure 2 represents the change in total n-3 LC-PUFA content of milk measured at 4-h intervals on days 1, 7, 14 and 21. Time 0 is the baseline level prior to taking the fish oil capsules for that day. Samples were then taken at 4, 8, 12 and 16 h postsupplementation. On day 1, LC n-3 PUFA content of the milk was significantly increased ($P = 0.0001$) by 8 h postsupplementation, and peak increases were observed at 12 h. The increase in milk LC n-3 PUFA resulting from fish oil ingestion continued on days 7 ($P = 0.02$), 14 ($P = 0.007$) and 21 ($P = 0.001$).

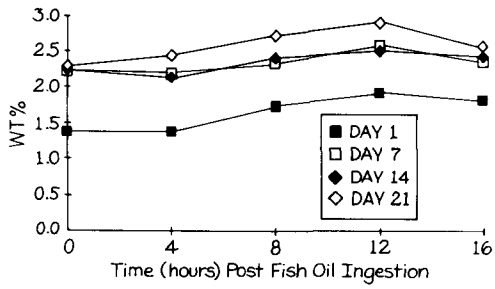

FIG. 2. Total n-3 polyunsaturated fatty acids (PUFA) content of human milk measured at four-hour intervals postsupplementation. Curves represent mean values (n = 5).

Erythrocyte fatty acid composition. The change in fatty acid composition of maternal erythrocytes is shown in Table 3. Significant increases were observed for EPA (583%) and DPA (150%) and for total n-3 PUFA (148%) contents. The n-6/n-3 fatty acid ratio was significantly decreased (77%) after fish oil supplementation. Although there was a trend toward increased DHA content (124%), this was not statistically significant. No significant changes were observed for the other fatty acids analyzed.

Infant erythrocyte fatty acid composition is shown in Table 4. Supplementation resulted in significantly increased EPA (636%) and DPA (260%) content, decreased n-6/n-3 fatty acid ratio (78%), and no change in DHA content (136%), similar to maternal changes.

Maternal platelet aggregation. Maternal platelet aggregation was measured in response to 1, 2 and 4 μM concentrations of ADP. Similar results were obtained at all ADP concentrations. Although there was no significant difference between mean measurements, some changes in response to fish oil supplementation were noted. Mean total aggregation response (%) to 4 μM ADP was 85 ± 12 on day 0, and 78 ± 3 on day 21. Total aggregation decreased 21% in subject 1, 7% in subject 2, 12% in subject 4, 5% in subject 5, and increased 10% in subject 3. Mean time to reach 50% aggregation was 24 ± 5 s on day 0 and 33 ± 12 s on day 21. Time to reach 50% aggregation doubled after supplementation in subjects 1 and 2, while subjects 3–5 showed little or no change.

Tocopherol content of human milk and plasma. No significant differences were observed in the tocopherol content of human milk or plasma from mothers or infants measured before and after the supplementation period. Mean α- and γ-tocopherol contents of milk and plasma are listed in Table 5.

TABLE 3

Change in Maternal Erythrocyte Fatty Acid Composition After Fish Oil Supplement[a]

Fatty acids	Day 0	Day 21
14:0	1.7 ± 2.4	0.5 ± 0.2
16:0	22.3 ± 2.5	20.4 ± 1.1
16:1	1.0 ± 1.1	0.1 ± 0.1
18:0	13.0 ± 2.2	14.3 ± 1.3
18:1	20.1 ± 6.7	17.2 ± 2.1
18:2n-6	8.2 ± 2.6	9.6 ± 1.2
18:3n-3	0.09 ± 0.06	0.03 ± 0.04
20:1	0.45 ± 0.11	0.43 ± 0.15
20:2n-6	0.45 ± 0.40	0.26 ± 0.06
20:3n-6	1.4 ± 0.54	1.5 ± 0.38
20:4n-6	12.9 ± 5.2	14.6 ± 1.3
20:5n-3	0.24 ± 0.16	1.4 ± 0.37[b]
22:1	0.35 ± 0.34	0.53 ± 0.35
22:4n-6	3.8 ± 1.4	4.1 ± 0.3
22:5n-3	1.4 ± 0.5	2.2 ± 0.3[c]
22:6n-3	4.5 ± 1.7	5.6 ± 1.1
Total PUFA	32.9 ± 11.3	39.2 ± 1.4
Total n-6	26.7 ± 9.2	30.0 ± 0.76
Total n-3	6.2 ± 2.2	9.2 ± 1.6[c]
n-6/n-3 ratio	4.3 ± 0.28	3.3 ± 0.56[b]

[a]Values are mean wt% ± SD (n = 5). PUFA, polyunsaturated fatty acids.

[b]Significantly different from day 0 ($P = 0.05$).

[c]Significantly different from day 0 ($P = 0.01$).

TABLE 4

Change in Infant Erythrocyte Fatty Acid Composition After Fish Oil Supplement[a]

Fatty acids	Day 0	Day 21
14:0	2.6 ± 3.7	0.84 ± 0.53
16:0	23.8 ± 3.1	20.9 ± 0.95
16:1	0.98 ± 1.3	0.16 ± 0.22
18:0	14.2 ± 1.5	15.4 ± 1.5
18:1	18.7 ± 6.0	15.2 ± 1.7
18:2n-6	5.6 ± 1.9	7.9 ± 0.33
18:3n-3	0.11 ± 0.15	—
20:1	0.26 ± 0.15	0.49 ± 0.08
20:2n-6	0.81 ± 0.22	0.58 ± 0.10
20:3n-6	1.7 ± 0.67	1.7 ± 0.21
20:4n-6	13.0 ± 6.2	15.0 ± 1.5
20:5n-3	0.11 ± 0.06	0.70 ± 0.34[b]
22:1	0.49 ± 0.33	0.47 ± 0.44
22:4n-6	3.4 ± 1.7	3.6 ± 0.63
22:5n-3	0.30 ± 0.15	0.78 ± 0.13[c]
22:6n-3	4.5 ± 2.2	6.1 ± 1.2
Total PUFA	29.5 ± 12.6	36.4 ± 3.3
Total n-6	24.5 ± 10.4	28.8 ± 1.9
Total n-3	5.1 ± 2.2	7.6 ± 1.5
n-6/n-3 ratio	5.0 ± 0.55	3.9 ± 0.66[b]

[a]Values are mean wt% ± SD (n = 5). PUFA, polyunsaturated fatty acids.

[b]Significantly different from day 0 ($P = 0.05$).

[c]Significantly different from day 0 ($P = 0.01$).

DISCUSSION

This study assessed the changes in human milk and maternal and infant erythrocytes in response to daily fish oil supplementation.

Analysis of human milk during fish oil supplementation. Fatty acids with chain lengths greater than C_{16} are derived from the diet and mobilized from adipose tissue stores. Because maternal weight remained stable during the study, it is assumed that the majority of milk fatty acids were derived from dietary sources (46).

In milk sampled at 4-h intervals on day 1, the n-3 VLC-PUFA content increased within 8 h after ingestion of the supplement. Peak levels occurred by 12 h and began to decline by 16 h. These results are in agreement with the findings of Hachey *et al.* (47) that peak fatty acid enrichment of breast milk occurred 8 to 10 h after consuming a labeled triglyceride meal.

Evidence indicates that the fatty acid composition of human milk does not exhibit diurnal variation (48–50). In the present study, samples taken at 4-h intervals had a significant rise in n-3 VLC-PUFA levels at 8 and 12 h postsupplementation on all days tested (see Fig. 2). It is not clear whether this within-day variation would persist if an equivalent level of n-3 VLC-PUFA was consumed in the form of a fish meal, or what effect smaller doses throughout the day would have on this variation. This observation is important to consider when planning a supplementation trial or comparing results between studies. Consistency in timing of sample collection is necessary for appropriate assessment and analysis of results.

Only one study has previously examined the change in fatty acid composition of human milk during fish oil supplementation. Harris *et al.* (28) supplemented 6 lactating women with 5 g of fish oil per day for 28 d. Samples were taken at only three time points, on days 0, 14 and 28. The authors also studied the effect of higher intakes for shorter time periods, again collecting only baseline, mid, and endpoint milk samples. Dose response-related increases were noted.

In the present study, daily milk samples were collected in order to formulate a model to characterize the change in fatty acid composition over the course of the supplementation period. The n-3 VLC-PUFA content increased most rapidly during the first and second day of supplementation, with smaller daily increases occurring between days 2 and 7. The n-3 VLC-PUFA content remained constant after day 7, at which point daily intake of the supplement served to maintain milk levels. There was a reciprocal decline in the n-6 VLC-PUFA content of the milk as n-3 fatty acid levels increased. Apparently, the synthesis and/or transport of n-6 VLC-PUFA were decreased by increased n-3 VLC-PUFA intake.

There was a decline in monounsaturated fatty acids and a significant increase in saturated fatty acids during the study period. These fatty acids did not exhibit response curves or knot estimates similar to those of the n-6 or n-3 fatty acids. Thus, it is not clear whether these changes were related to the supplementation trial or due to other factors, such as the stage of lactation. There is some evidence that 14:0 is increased, and 18:1 is decreased as lactation progresses (51), although Clark *et al.* (38) found that the fatty acid composition of mature milk remains constant between 2 and 16 wk *postpartum.*

Data from various studies indicate that human milk usually contains 0.03–0.2% EPA, trace–0.5% DPA and

TABLE 5

Tocopherol Status Before and After Fish Oil Supplement[a]

	α-Tocopherol		γ-Tocopherol	
Sample	Day 0	Day 21	Day 0	Day 21
Human milk (μg/L)	1779 ± 804	1592 ± 522	886 ± 290	1032 ± 439
Maternal plasma (μg/mL)	1.50 ± 0.6	1.49 ± 1.1	3.75 ± 1.1	3.48 ± 1.0
Infant plasma (μg/mL)	1.71 ± 1.9	4.17 ± 3.0	1.72 ± 0.3	2.06 ± 0.7

[a]Values are mean ± standard deviation (SD) (n = 5).

0.1-0.6% DHA (17,19,20,52). The present study is the first to calculate the dietary intake of n-3 VLC-PUFA. Our data show that mean breast milk composition from women consuming about 1 g of total n-3 PUFA, with 120 mg from n-3 VLC-PUFA, was 0.08% EPA, 0.14% DPA and 0.37% DHA.

A comparison of the n-3 fatty acid composition of human milk from women supplemented with fish oil (29) to milk from Inuit women naturally consuming increased levels of n-3 VLC-PUFA (28) indicates that supplementation with over 10 g of fish oil is necessary to produce n-3 VLC-PUFA levels comparable to those found in Inuit breast milk. Recommendations to increase fish consumption to this level are impractical in our society, and the optimal level for health benefits and infant development may not need to be this high. Studies regarding the effect of maternal dietary changes on infant tissue fatty acid composition are crucial to determine dietary requirements for lactating women as well as for making appropriate recommendations regarding the composition of infant formulas.

Erythrocyte fatty acid composition. This study was the first to examine the change in fatty acid composition in response to maternal fish oil supplementation. It is interesting to note the large increase in EPA, the doubling of DPA, with no significant change in DHA content (Tables 3 and 4). There was no change in the n-6 fatty acid composition. These findings are in agreement with studies in adults, where fish or fish oil consumption resulted in a much greater increase in EPA than DHA in erythrocyte phospholipids, while 18:2n-6 and 20:4n-6 did not change or decreased only slightly (32).

The high level of DHA found in brain and retinal phospholipids is thought to be important to the physical properties of membranes in these tissues. The membrane content of DHA is tenaciously maintained in the face of deficiency (5). Subjects in this study consumed only 33% more EPA than DHA and a small amount (22 mg) of DPA per day (Table 1). The lack of increase in DHA content, with correspondingly large increases in EPA and DPA, suggests that retroconversion of DHA to EPA and DPA may be occurring. This phenomenon has been demonstrated in platelet phospholipids (53) and is suggesetive of a regulatory mechanism which controls the level of DHA in cell membranes.

Increased tissue content of EPA is thought to be an important modulator of eicosanoid metabolism with potential cardiovascular benefits. The level required to confer such benefits without adverse effect remains uncertain.

Maternal platelet aggregation response. This is the first study to examine platelet aggregation in lactating women. Total aggregation response was similar to the mean of 78 ± 11% observed by Roper *et al.* (54) in 51 females. Some changes in platelet responsiveness were noted, although mean differences were not statistically significant. Decreased aggregation response was expected based on previous studies using similar dosages of fish oil for 3-4 wk (55,56), although Boberg *et al.* (57) also found no significant difference in ADP-induced platelet aggregation response after fish oil supplementation. The clinical relevance of the changes observed in this study remains to be determined.

Tocopherol content of diets, milk and plasma. Although dietary vitamin E was found to be below the recommended daily allowance (RDA) of 12 mg during lactation, only the α-tocopherol content of foods was calculated for this study, thus there was an underestimate of dietary tocopherol intake. As the tocopherol content of foods and daily intakes are widely variable and the available databases are incomplete with regard to the tocopherol content of foods, it is difficult to truly assess the adequacy of vitamin E intake in this study. Others have also found dietary intake to be below the RDA for vitamin E (58,59), although subjects maintained adequate plasma levels of vitamin E (59).

The tocopherol content of the milk sampled in this study was within the range observed by others (60-62). Maternal plasma α-tocopherol levels were lower than levels observed by others (63,64). This may be a manifestation of the low dietary intake noted in this study. Alternately, data regarding plasma tocopherol status during lactation are lacking, and levels may be different from nonlactating women. Infant plasma tocopherol was also somewhat lower than the 6.89 μg/mL observed in 4-15 d-old infants (65), despite comparable milk levels.

As no changes in tocoperhol levels were noted, this suggests that vitamin E deficiency is not induced by fish oil supplementation. However, fish oil supplementation provided mothers with an additional 6 mg of vitamin E per day, and the significance of this contribution to dietary intake was not determined. Knapp *et al.* (66) also found no change in serum α-tocopherol during fish oil supplementation.

Conclusion. Maternal fish oil supplementation increased the n-3 VLC-PUFA and decreased the n-6 VLC-PUFA contents of human milk. One week was required to reach steady state levels. Increases in milk n-3 VLC-PUFA were observed within 8-h postsupplementation, and a diurnal variation existed throughout the study. Maternal and infant erythrocyte fatty acid compositions were similarly affected with significant increases in EPA and DPA but no change in DHA or n-6 PUFA contents. No differences in mean tocopherol values or maternal platelet aggregation responses were noted.

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Positional Specificity of Gastric Hydrolysis of Long-Chain n-3 Polyunsaturated Fatty Acids of Seal Milk Triglycerides[1]

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Long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) of marine oils are important dietary components for both infants and adults, and are incorporated into milks following maternal dietary intake. However, little is known about the hydrolysis of these PUFA from milk triglycerides (TG) by lipases in suckling young. Seals, like humans, possess gastric lipase; however, the milk lipids of seals and sea lions are almost devoid of the readily hydrolyzable medium-chain fatty acids, and are characterized by a large percentage (10–30%) of n-3 PUFA. Gastric hydrolysis of milk lipids was studied *in vivo* in suckling pups of three species (the California sea lion, the harp seal and the hooded seal) in order to elucidate the actions and specificity of gastric lipases on milk TG in relation to fatty acid composition and TG structure. Regardless of milk fat content (31–61% fat) or extent of gastric hydrolysis (10–56%), the same fatty acids were preferentially released in all three species, as determined by their relative enrichment in the free fatty acid (FFA) fraction. In addition to 16:1 and 18:0, these were the PUFA of 18 carbons and longer, except for 22:6n-3. Levels of 20:5n-3 were most notably enriched in FFA, at up to five times that found in the TG. Although 22:6n-3 was apparently also released from the TG (reduced in the diglyceride), it was also notably reduced in FFA. Positional analysis of milk TG based on the products of Grignard hydrolysis revealed that these PUFA, including 22:6n-3, were preferentially esterified at the α-position of the TG, and that the fatty acids not released during gastric hydrolysis were located at the *sn*-2 position. The extreme reduction of 22:6n-3 and enrichment of 20:5n-3 in FFA is discussed. Results from this study are consistent with reports that gastric lipase acts stereospecifically to release fatty acids at the α-positions (*sn*-3, *sn*-1). We conclude that the n-3 PUFA in milk are efficiently hydrolyzed by gastric lipase and that this has important implications for digestion of milks enriched in PUFA by neonates in general.
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Long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), characteristic of marine oils, have become a major focus of research due to their potent antiatherogenic (1,2), antineoplastic, and antiinflammatory (3) effects. Recent evidence suggests their intake to be important from an early age for these reasons (*i.e.*, eicosanoid synthesis), as well as for normal neonatal brain and nervous development and cell membrane structure (4–7). Long-chain n-3 PUFA are readily incorporated into milks of various species following maternal dietary intake (8,9). However, information on the hydrolysis and absorption of these n-3 PUFA in milks by suckling young is limited.

Milk lipid is composed primarily of triglyceride (TG; 97–99%) and, in order to be absorbed in the intestine, must be hydrolyzed to free fatty acid (FFA) and monoglyceride (MG). Although lipids are essential to the neonate for both normal growth and metabolism, in species that have been studied (*e.g.*, human, rat, dog), intestinal function is immature, resulting in reduced levels of pancreatic lipase and bile salts (10–12), both of which are required for the intestinal digestion of fat. Additionally, it is reported that pancreatic lipase, which is specific for the α-positions (*sn*-1 and *sn*-3) of TG, does not hydrolyze TG ester bonds containing long-chain n-3 PUFA (13,14). In fish oils, n-3 PUFA are esterified primarily at the *sn*-2 position of the TG and thus can be readily absorbed as *sn*-2 MG upon pancreatic lipase action (15–18). However, the n-3 PUFA in depot fats of mammals are located primarily at the *sn*-3 and secondarily at the *sn*-1 positions (18–21), and thus whale oil TG have been found to be resistant to pancreatic lipase (22).

Thus, in view of these relative inadequacies, fat absorption in newborns may depend upon compensatory or alternative mechanisms of hydrolysis by means of preduodenal lipases. In particular, initial intragastric lipolysis by lingual and gastric lipases may be especially important for the digestion of milk fat, which is packaged as globules and is otherwise resistant to pancreatic lipase (23,24). However, studies of lipolysis by gastric and lingual lipases have been based largely on *in vitro* studies using a single component fatty acid in artificial lipid emulsions and have stressed their preferential release of medium-chain fatty acids (MCFA). While milks of some species contain notable proportions of MCFA, milk lipids of humans and most carnivores are composed mostly of long-chain fatty acids (25–27). Milk lipids of seals and sea lions furnish 85–95% of the total caloric intake of neonates and are characterized primarily by long-chain and unsaturated fatty acids, including a very large percentage (10–30%) of n-3 PUFA derived from their marine diets (28,29). Suckling pups consume phenomenal quantities of lipid daily at 600 g to 5 kg (30,31), or up to 10- to 20-fold higher on a body weight basis than neonates of the human or dog. Rapid blubber deposition suggests rapid and efficient absorption of milk lipid (28,32,33), but the mechanisms of hydrolysis and digestion are unknown. Gastric lipase is present in seal pups (34); however, milks are virtually devoid of the readily hydrolyzable MCFA. These species thus provide an excellent opportunity to elucidate the actions of gastric lipases on milk TG in relation to fatty acid composition and TG structure.

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Abbreviations: BHT, butylated hydroxytoluene; DG, diglyceride; FFA, free fatty acid; GC, gas chromatograph; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; MCFA, medium-chain fatty acid; MG, monoglyceride; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TG, triglyceride; TLC, thin-layer chromatography.

In this study we examined milk lipid hydrolysis by gastric lipase in suckling neonates of the harp seal *(Phoca groenlandica),* the hooded seal *(Cystophora cristata)* and the California sea lion *(Zalophus californianus).* Our objectives were to characterize the location of the n-3 PUFA in seal milk TG by positional analysis using Grignard hydrolysis (35) and to measure *in vivo* hydrolysis during intragastric digestion. We used data on the milk TG structure and comparison of the proportions and compositions of hydrolysis products, produced during both gastric lipase hydrolysis and Grignard hydrolysis, to elucidate the actions and stereospecificity of gastric lipase and the digestibility of n-3 PUFA in milk.

MATERIALS AND METHODS

Sample collection. Samples were collected from mothers and pups of harp seals and hooded seals on the pack ice off the southeast coast of Labrador, Canada, and from California sea lions on San Nicolas Island, Channel Islands, California. Milk was manually expressed from chemically immobilized females following administration of oxytocin. Prior to analysis, milk samples were stored frozen at −20°C (sea lion) or frozen in chloroform/methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene (BHT) (harp and hooded seals) from the time of collection.

Pups were physically restrained and stomach contents (ingested milk) were evacuated by intubation with a 3/8 inch veterinary stomach tube, opportunistically in harp (n = 15) and hooded seals (n = 17), or at timed intervals following observed suckling in sea lions (n = 20). At the time of collection, all samples were placed in chloroform/methanol (2:1, vol/vol) with BHT and stored frozen at −20°C until analysis. Characterization of gastric hydrolysis (analysis of milk TG and hydrolysis products) was investigated in five mothers and pups of each species.

Neutral lipid class analysis and separation. All solvents used were HPLC (high-performance liquid chromatography) grade or analyzed reagent grade and all containers and equipment were composed of glass or teflon and pre-washed with solvent prior to use. Samples were extracted using a modification (25,28) of the method of Folch *et al.* (36).

Proportions of neutral lipid classes were determined in milk and gastric samples by thin-layer chromatography (TLC) and quantitative densitometry. Plates used for TLC were commercial 20 × 20 cm, precoated (Si250-PA) channeled glass plates (Baker Chemical Co., Phillipsburg, NJ) with a preadsorbent spotting area. Plates were predeveloped in 100% diethyl ether in order to remove the organic binder which typically runs at the solvent front, and were activated at 120°C for 20 min before spotting. A two-stage development process was employed using chloroform/methanol/ethanol/acetic acid (98:2:1:0.1, by vol) (partial plate development to 17 cm) followed by hexane/diethyl ether/acetic acid (94:6:0.2, vol/vol/vol) (37). Plates were charred in a temperature-programmed oven after dipping in 10% cupric sulfate in 8% phosphoric acid (38). Quantitative spectrodensitometry was performed using a Shimadzu CS-910 Dual-Wavelength TLC scanner (Shimadzu Scientific Instruments, Inc., Columbia, MD) using a linear scanning mode (39). Peak areas were automatically integrated and lipid concentrations were calculated from standard curves fitted by least squares regression from a range of standards run each day of analysis.

Individual neutral lipid classes (TG, α,α- and α,β-diglyceride (DG), α- and β-MG and FFA) were separated for subsequent fatty acid analysis as follows. Aliquots of the initial chloroform extract containing approximately 40 mg lipid were spotted on predeveloped activated (1 h at 100°C) preparative TLC plates. Plates were 20 × 20 cm glass, coated with silica gel G-60 (E. Merck, Elmsford, NY) at 0.5 mm thickness. A standard lane was spotted on each plate with a mixture of neutral lipid standards. Plates were developed in the same 2-solvent system described above. Lipid classes were visualized and marked under UV light after spraying lightly with 0.2% 2′,7′-dichlorofluoresceine in absolute ethanol (w/v). Silica gel areas containing the different classes were carefully scraped onto aluminum foil using a teflon spatula and transferred into separate glass tubes for subsequent transesterification.

Positional analysis. After initial comparison of results obtained from methods outlined by Brockerhoff (40) and Christie (41), the following procedure was used to hydrolyze TG to α,α-DG, α,β-DG, α-MG and β-MG. Anhydrous diethyl ether (5 mL) was added to approximately 50 mg of mammary milk TG and the sample was set in an ice bath. To this was added 0.5 mL of ethyl magnesium bromide (Grignard reagent) (3.12 M in diethyl ether, Alfa Chemicals, Morton-Thiokol Inc., Davers, ME). The mixture was then agitated for 4 min, after which 0.2 mL glacial acetic acid and 1 mL H_2O were added to stop the reaction, followed by 1 mL aqueous potassium bicarbonate (2%). The tube was shaken, centrifuged, and the lower layer (H_2O) pipetted out. The remaining ether layer was washed an additional three times with H_2O, removing as much H_2O as possible at each wash. Anhydrous sodium sulfate was then added to dry the ether extract. Hydrolysis products were separated by TLC on silica gel (G-60) impregnated with 5% (w/w) boric acid and developed in chloroform/acetone (96:4, vol/vol) (42). Fractions were visualized and isolated as described above for subsequent transesterification.

Preparation of fatty acid methyl esters. Transesterification and subsequent extraction of methyl esters were performed in the presence of the silica after initial verification that the presence or absence of silica did not alter fatty acid patterns. To each sample was added 1.5 mL of methylene chloride with 0.01% BHT and 3.0 mL Hilditch reagent (0.5N H_2SO_4 in methanol); the sample was flushed with nitrogen and stored in a glass tube with teflon-lined cap in the dark at room temperature for 72 h. This method resulted in similar recoveries of methyl esters as were obtained using methanolic HCl at high temperatures, and was considered preferable due to the elimination of the risks of oven heating effects on PUFA, as well as the absence of artifact production. Alkali isomerizarion is reported with basic reagents (41).

Following transesterification, methyl esters were extracted into hexane. Hexane extracts were then concentrated to about 0.25 mL under nitrogen at 30°C, applied to thin-layer plates and purified by preparative TLC using a solvent system of light petroleum ether (30–60°C boiling point)/diethyl ether (95:5, vol/vol) (43). Methyl esters were visualized and collected from the silica gel by eluting with five column volumes of methylene chloride in a glass column plugged with glass wool. The sample was then concentrated under nitrogen, and isooctane was added at approximately 25 mg lipid/mL isooctane.

Gas-liquid chromatography. Fatty acid analyses were performed by temperature-programmed gas-liquid chromatography (GLC) on a Hewlett-Packard (Avondale, PA) Model 5880 Gas Chromatograph (GC) fitted with a Monarch series flexible glass capillary column (25 m × 0.25 mm i.d.) according to the methods of Sampugna *et al.* (43). The column was coated with SP-2340 (Quadrex Corp., New Haven, CT) at 25 μ film thickness. The carrier gas was helium and the make up gas was nitrogen. The GC was equipped with a flame ionization detector (temperature 275°C). The temperature of the injection port was maintained at 250°C. Following sample injection, the temperature of the column was held at 153°C for 2.0 min, then programmed to increase to 174°C at the rate of 2.25°C per min, then to increase to 200°C at the rate of 2.5°C per min and held there until the end of the run. The entire run time was approximately 24 min.

Identifications of fatty acid methyl ester peaks were based on retention times of known standard mixtures run daily and also after isolation of fatty acid classes in selected samples from each species using silver nitrate ("argentation") chromatography (28). Methods for silver nitrate chromatography essentially followed recommendations by Christie (41), except that a mobile phase of hexane/diethyl ether (90:10, vol/vol) was used to separate methyl esters containing zero to two double bonds, hexane/diethyl ether (40:60, vol/vol) to separate three to six double bonds and, finally, petroleum ether/diethyl ether/acetic acid (90:8:2, vol/vol/vol) to separate zero to six double bonds for comparison. Peak areas were measured with an electronic integrator and normalized by individual response factors obtained from available standards. Sample injection volume was usually 0.3 μL, or about 7.5 μg lipid. Fatty acids are designated by shorthand nomenclature of chain length:number of double bonds with (n-x) denoting the position (x) of the last double bond relative to the terminal methyl end.

Calculations and statistical analyses. The relative weight contribution (*i.e.*, molecular weight) of individual fatty acid methyl esters differs greatly according to chain length and degree of unsaturation. Therefore, in the comparison of hydrolysis products to that of TG and in the positional analyses, fatty acid composition was expressed on a mol % basis rather than wt %, unless otherwise indicated. The average molecular weight used in calculations for each component (TG, DG, MG and FFA) was based on their actual fatty acid compositions as determined from GLC analysis. The extent of hydrolysis in gastric samples was defined as the disappearance of TG (or increase in sum of hydrolysis products) from that originally present in milk.

Although 40–50 fatty acids (including isomers) were routinely observed in samples, fatty acid profiles were summarized according to the 14 major representative fatty acids of marine oils and seal lipids (28,44). Because all isomers of a given monounsaturated component behaved identically in this study, isomers were combined and expressed as a single value.

The mol % composition of the *sn*-2 position of milk TG was calculated from Grignard hydrolysis data as:

$$[sn\text{-}2] = 4\ [\alpha,\beta\text{-DG}] - 3\ [\text{TG}] \qquad [1]$$

(*e.g.*, refs. 40,41). For each fatty acid, the percent of the total amount present in milk which was located at the *sn*-2 position was then:

$$\%\ @\ sn\text{-}2 = \frac{[sn\text{-}2]}{3\ [\text{TG}]} \times 100 \qquad [2]$$

The average composition of the α-positions (*sn*-1, *sn*-3) was calculated as:

$$[sn\text{-}1,\ sn\text{-}3] = \frac{3\ [\text{TG}] - [sn\text{-}2]}{2} \qquad [3]$$

Calculations were performed using values obtained for α,β-DG rather than for α,α-DG since the latter tend to be more highly contaminated by acyl migration of α,β-DG components (40).

Data are presented as mean ±SEM. Unless otherwise indicated, all percentage values are mol %. Statistical analyses were performed using Statview 512+ (Brain-Power Inc., Calabasas, CA) for the Macintosh.

RESULTS

Neutral lipid class composition and evidence of gastric hydrolysis. Milk TG composition and extent of gastric lipolysis were determined for each species (Table 1). Milk lipids of harp and hooded seals, stored in solvents from the time of collection, were composed of greater than 99% TG, with very minor amounts of FFA (0.0–0.1%), DG

TABLE 1

Gastric Hydrolysis of Milk Lipid in Seal Neonates[a]

	Milk fat (wt %)	TG in milk (mol %)	n	Gastric hydrolysis (mol %)		
				Average[b]	Maximum	n
Sea lion	31.7[c]	98.4 ± 0.06	(4)	9.3 ± 1.33	25%	(20)
Harp seal	51.8[d]	99.6 ± 0.05	(5)	10.1 ± 2.65	37%	(15)
Hooded seal	61.0[e]	99.4 ± 0.95	(5)	23.0 ± 3.24	56%	(17)

[a] Average values are means ± SEM. TG, triglycerides.

[b] Average values taken from mid-sampling point in sea lions (28) and from all animals sampled in harp and hooded seals.

[c] From ref. 31.

[d] From ref. 30.

[e] From ref. 45.

(0.2–0.5%) and cholesterol (0.1–0.2%). Sea lion milks (n = 4 samples which had not undergone thawing and refreezing), although stored frozen for six years without solvents prior to analysis, showed similarly high levels of TG (98.4%). Although amounts of other components were sightly higher (0.2% FFA, 0.8% DG and 0.1% MG), evidence of hydrolysis during storage was minimal. Cholesterol ester was not detected in any sample.

The comparison of neutral lipid class composition of gastric samples from pups revealed that substantial amounts of hydrolysis occurred in all species (Table 1). The average level of hydrolysis was at least twofold higher in the hooded seal than in the harp seal and sea lion and corresponded to the higher levels of total milk fat. Milk TG levels were found to drop as low as 43.6%, 62.8% and 75.0% in the hooded seal, harp seal and sea lion, respectively. In all cases, the primary products of hydrolysis were α,β-DG (0.2–20.0%) and FFA (0.0–31.2%). Small amounts of MG (0.0–4.4%) and only trace amounts of α,α-DG (0.0–1.1% in harp and hooded seals only) were occasionally present.

Positional analysis. Positional analysis by Grignard hydrolysis of milk TG of all three species revealed distinct differences in the distributions of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) as groups (Fig. 1). SFA tended to be very enriched at the *sn*-2 position of the milk TG, which contained essentially 70–90% of all SFA occurring in milks (see equation 2). By contrast, MUFA and PUFA were reduced at the *sn*-2 (Fig. 1), indicating their tendency to be esterified to the α-position. In all three species, 13–27% of the MUFA in milk were at the *sn*-2 position, while only 2–11% of all PUFA were at *sn*-2.

Grignard hydrolysis data also provided information on the composition of individual fatty acids at the *sn*-2 position in comparison to that of the intact milk TG (Fig. 2). Specifically, the major fatty acids enriched at the middle position in seal milk TG were the shorter-chain saturates 14:0 and 16:0. The other principal SFA, 18:0, was enriched at *sn*-2 in the hooded seal but reduced at *sn*-2 in the harp seal and sea lion (not illustrated); however, it was less than 2% of total fatty acids and therefore absolute differences were not large. The other major fatty acid which was always enriched at *sn*-2 was 16:1.

The MUFA 18:1 was reduced at *sn*-2 in comparison to the intact TG, but absolute levels at *sn*-2 were still high; thus 16:1 and 18:1 contributed to most of the measurable MUFA at *sn*-2 (Fig. 1). The other MUFA, 20:1 and 22:1, as well as all major PUFA, were virtually absent from the *sn*-2 position (Fig. 2), indicating that all were located at the α-position of the TG. Of the 14 major marine oil fatty acids, the PUFA 18:2n-6, 18:3n-3, 18:4n-3 and 20:4n-6 were variably reduced (or enriched) at *sn*-2, but are not illustrated in Figure 2 as these were generally less than 1.5% of total fatty acids and, therefore, absolute amounts and differences were very small. All three species exhibited the same patterns.

Fatty acid composition of milk TG and gastric hydrolysis products. Five mother/pup pairs were chosen for this part of the study to account for the variability in fatty acid compositions found among individual milks. Because some of the gastric samples from pups of each species had very low levels of hydrolysis, it was not always possible to obtain individual lipid classes (FFA, DG, MG) in sufficient quantity for fatty acid analysis of each class. Hence n differed from five in these cases, since composition of milk TG could only be compared to the FFA, DG and MG produced from the same milks after gastric digestion.

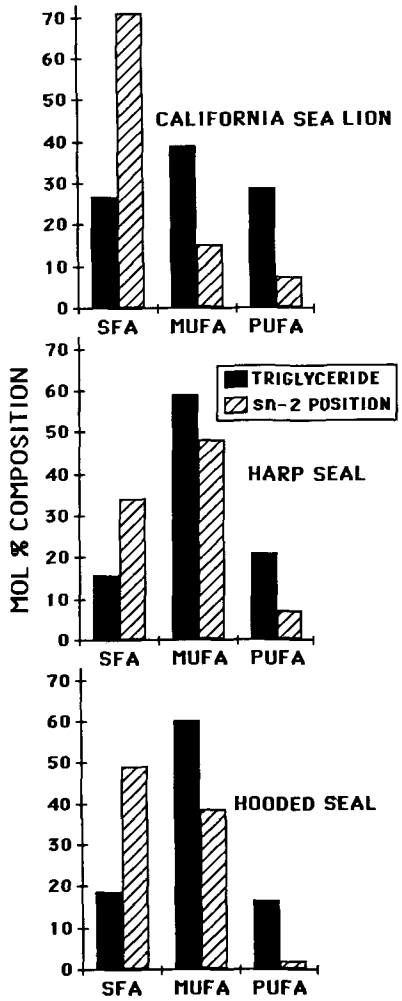

FIG. 1. Positional distribution of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) in the triglyceride and at the *sn*-2 position in milk of three seal species as determined from Grignard hydrolysis. Mol % composition of fatty acid groups at *sn*-2 position was calculated as: [*sn*-2] = 4 [α,β-DG] − 3 [TG].

Analyses of the fatty acid composition of milk TG and the FFA released during gastric hydrolysis revealed highly significant differences between all components in each species ($P < 0.01$, paired-t, Fig. 3). The FFA were significantly reduced in the SFA 14:0 and 16:0, as well as in the MUFA 18:1, 20:1 and 22:1. In fact, the fatty acids preferentially released during gastric digestion, other than 18:0 and 16:1, were the long-chain PUFA, as determined by their significant enrichment in FFA. The n-3 PUFA 20:5n-3 was extraordinarily enriched, at up to five times the level in the TG. The single exception was 22:6n-3, which was surprisingly greatly reduced in FFA of all species (Fig. 3), at levels as low as one-sixth of that found in TG.

In examination of the composition of the other gastric hydrolysis products, as expected, the specific fatty acids

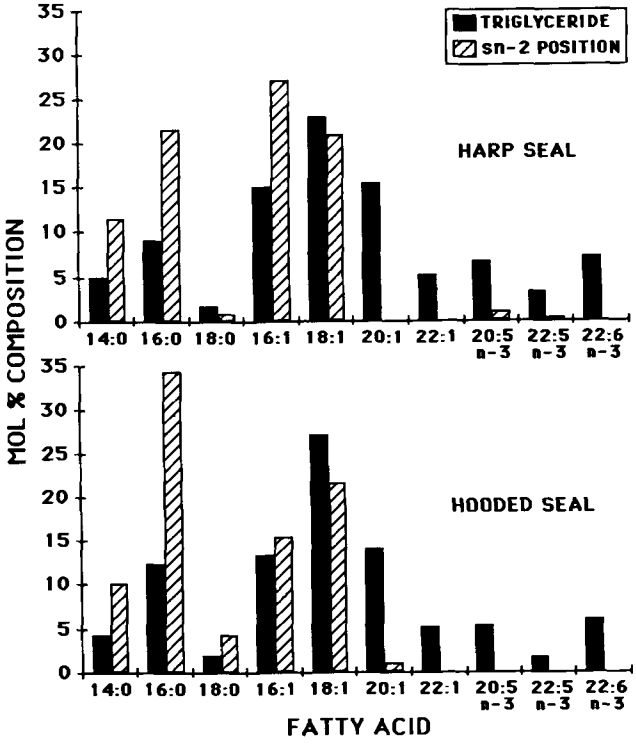

FIG. 2. Fatty acid composition of the triglyceride and at the *sn*-2 position in seal milk as determined from Grignard hydrolysis. Mol % composition of individual fatty acids at *sn*-2 position was calculated as: [*sn*-2] = 4 [α,β-DG] − 3 [TG]. Of the 14 major marine oil fatty acids, the PUFA 18:2n-6, 18:3n-3, 18:4n-3 and 20:4n-6 are not illustrated as these were usually less than 1% of total fatty acids. Data for the sea lion are not presented but exhibited the same patterns.

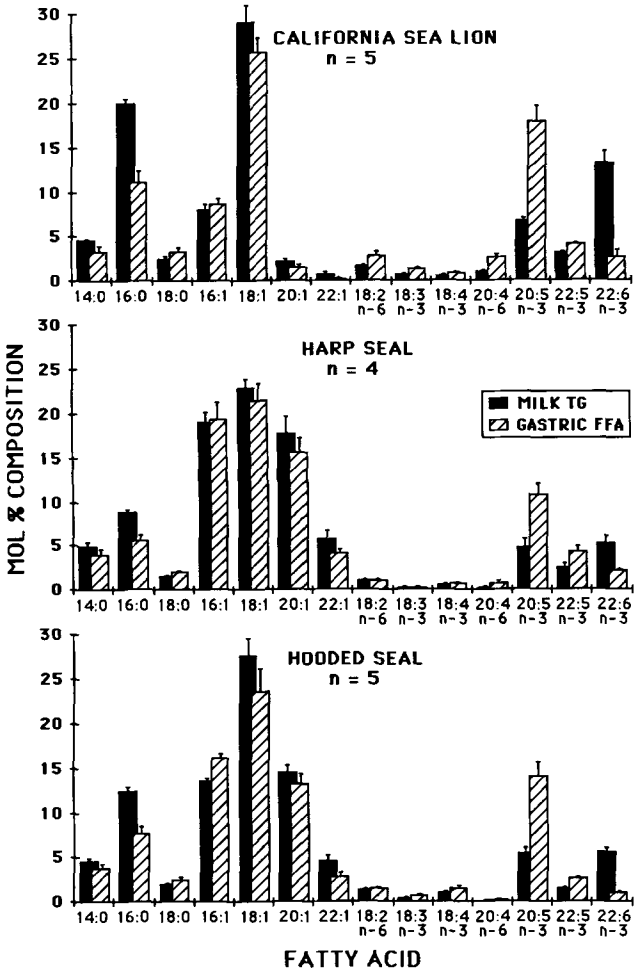

FIG. 3. Fatty acid composition of maternal milk TG and the FFA subsequently released during intragastric hydrolysis in pups of three seal species. Bars are mean mol % composition and vertical lines are + 1 SEM. Differences in fatty acid composition between TG and gastric FFA were in the same direction for all species and were significant for all fatty acids ($P < 0.01$, n=14, paired-t).

which were reduced in FFA were found enriched in the DG compared to the TG. Likewise, those fatty acids found at enriched levels in FFA were found at reduced levels in DG. In other words, the fatty acids which were preferentially released from the TG consequently left the DG reduced (*e.g.*, 20:5n-3 and 22:5n-3, Fig. 4). However, once again the single exception was 22:6n-3, which was reduced in DG (indicating its release from the TG), but also greatly reduced in the FFA (Fig. 4). In spite of differences in absolute levels of hydrolysis and fatty acid compositions, the exact same patterns occurred in all three species.

The levels of individual fatty acids in the MG obtained from gastric hydrolysis accentuated the trends found in the DG in that the fatty acids enriched in DG as compared to TG were further enriched in MG and those reduced in DG were further reduced in MG. The MG obtained in gastric samples was almost exclusively *sn*-2 MG, whereas Grignard hydrolysis produced both *sn*-2-MG and α-MG, which were difficult to separate on preparative thin-layer plates and also likely to be cross-contaminated by acyl migration. Thus the gastric MG were considered to be truly representative of the *sn*-2 position. Hence, these data were compared to both the estimated *sn*-2 position, as determined from Grignard hydrolysis, as well as to the average composition of the α-position of the milk TG (Fig. 5). In fact, in all species, the fatty acid composition of the MG obtained following gastric hydrolysis *in vivo* was remarkably similar to the estimated *sn*-2 composition calculated from the positional analysis using Grignard α,β-DG. Conversely, the α-position was in marked contrast to both estimates of the 2-position; in comparison, the α-position was particularly reduced in 14:0, 16:0 and 16:1 and highly enriched in the MUFA, 20:1 and 22:1, and the n-3 PUFA (Fig. 5).

DISCUSSION

Milk lipid is the principal source of energy for suckling neonates of most mammalian species. Even in human milk at about 4% fat, it provides greater than 50% of total calories. More significantly, it is the sole source of essential and n-3 PUFA for the rapidly developing suckling neonate. Recent studies have shown that when maternal dietary intakes of long-chain PUFA are elevated, milk lipids become readily enriched in these fatty acids (8,9,46). However, the actual absorption and utilization of these fatty acids by the neonate depends upon initial hydrolysis.

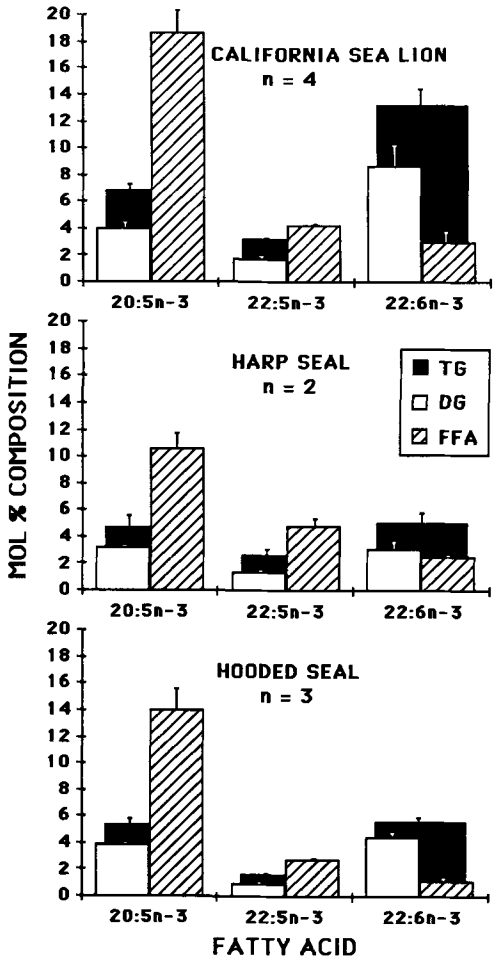

FIG. 4. Composition of the long-chain n-3 PUFA in maternal milk TG and in the hydrolysis products (DG and FFA) released during intragastric hydrolysis in pups of three seal species. Bars are mean mol % composition and vertical lines are +1 SEM.

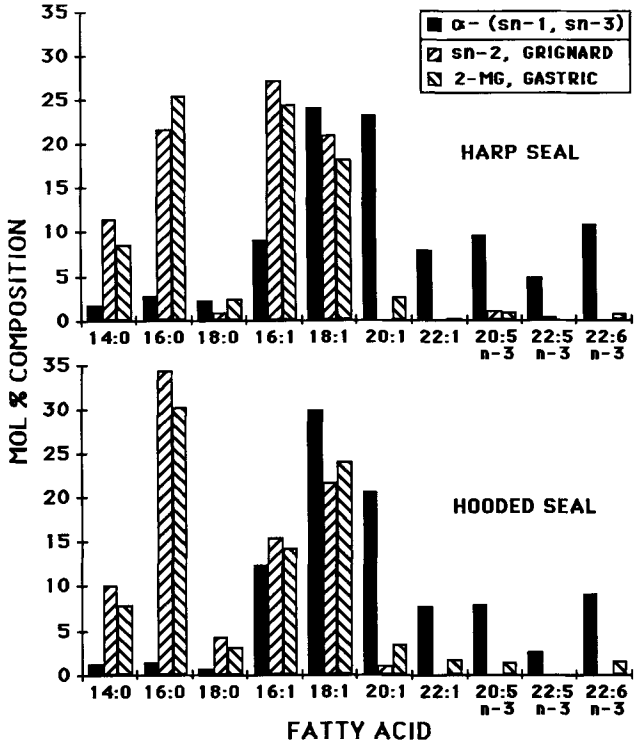

FIG. 5. The average fatty acid composition of the α-positions (*sn*-1, *sn*-3) (from positional analysis), in comparison to the estimated ("Grignard") *sn*-2 position (from positional analysis) and the *sn*-2 MG obtained following intragastric hydrolysis. Average mol % composition of the α-positions was calculated as: [*sn*-1, *sn*-3] = (3 [TG] − [*sn*-2])/2. Mol % composition of the *sn*-2 position (Grignard) was calculated as: [*sn*-2] = 4 [α,β-DG] − 3 [TG]. Data for the sea lion are not presented but exhibited the same patterns.

The importance of initial gastric digestion of dietary lipid by lingual and/or gastric lipases, especially in the neonate, has become increasingly evident (47). However, most information on hydrolysis has been limited to artificial homogeneous TG containing fatty acids of chain-length and degree of unsaturation of 18:1 or lower. Neonates of the three species of seals used in this study are similar to humans, some carnivores and the rabbit (48–52), in that they possess gastric lipase as the primary preduodenal lipase acting in the stomach (28,34). The study of gastric hydrolysis of lipid-rich seal milks (Table 1), which contain high proportions of long-chain PUFA, may provide a better understanding of the digestion of the n-3 PUFA in milks in general and of the stereospecificity of gastric lipases.

Lipases typically exhibit specificity for substrate, position and/or fatty acid. Gastric and lingual lipases, which are analogous in both structure and function (53–57), are reported to exhibit considerable stereospecificity for the α-position of the TG and to hydrolyze the *sn*-3 position twice as fast as the *sn*-1 position (23,58–61). In this study, the primary products of hydrolysis were α,β-DG and FFA in all three seal species. The occurrence of α,β-DG as virtually the sole DG product and the predominance of DG rather than MG as gastric hydrolysis products is consistent with the stereospecificity of gastric lipase. The occurrence of trace amounts of α,α-DG was probably due to small amounts of acyl migration. The *sn*-2 position did not appear to be hydrolyzed.

Thus, stereospecific analysis of TG and hydrolysis products provides some clues about the mechanisms of gastric digestion. Although pancreatic lipase hydrolysis is often used for positional analyses, it is not appropriate for TG which contain long-chain PUFA in the primary positions since pancreatic lipase is specific for the α-position, but does not hydrolyze long-chain PUFA, such as 20:5n-3, 22:5n-3 and 22:6n-3 (13,14,41). In contrast, Grignard reagent (ethyl magnesium bromide) releases the fatty acids (as tertiary alcohols) from all three positions of TG, generating intermediate products, which include α,β- and α,α-DG. In this study, Grignard hydrolysis tended to produce some breakdown products within classes which were recorded as unidentified peaks during GLC analysis and excluded from calculations. The cleanest products were the α,β-DG. Because α,β-DG produced during chemical hydrolysis also tend to be free of acyl migration (41), these were used for calculations of the 2-position.

Previous studies have shown that the positional distribution of specific fatty acids in milk TG tend to be very similar across a wide range of mammalian species (7,40,

62,63); however, the distribution of long-chain and n-3 PUFA in milks has rarely been studied. The positional data obtained in this study are comparable to those found in other marine mammal species (Table 2). In all species, the *sn*-2 position of milk TG contained about 70–100% of the 14:0 and 16:0 present in milk (Table 2 and Fig. 2), a finding characteristic of the milks of many mammalian species, including human, pig and dog (7,62,63). This distribution is consistent with the observed action of gastric lipase in seal neonates. Preferential hydrolysis of the α-position by gastric lipase would explain the paucity of 14:0 and 16:0 in FFA (Fig. 3) and the relative accumulation of these fatty acids in DG. Similar accumulations of 14:0 and 16:0 have been reported in rat milk DG and MG following gastric hydrolysis (66).

In all mammalian species previously investigated, 18:0 has been found selectively esterified at the *sn*-1 position with roughly 9–11% occurring at the *sn*-2 (62,63). Similar proportions of 18:0 have been found at the *sn*-2 in most seal and whale milks (Table 2), thus the slight enrichment of 18:0 in gastric FFA in this study (Fig. 3) may have been due to accumulation from hydrolysis of both *sn*-1 and *sn*-3. However, the hooded seal is exceptional in that selective esterification (74%) appears to occur at *sn*-2 (Table 2), suggesting that some aspects of milk TG structure may actually differ among species, as has previously been indicated in selected species (63).

In marine mammals, as in virtually all other species studied (62,63), 16:1 tends to be evenly distributed at all positions of the milk TG, whereas 18:1 tends to be more enriched at the α-positions. As expected, 16:1 and 18:1 are readily hydrolyzed during gastric lipase action. In the Weddell seal, 16:1 is more prevalent at *sn*-3 than at *sn*-1, whereas the reverse is true for 18:1 (63), which may account for the slight enrichment and reduction in FFA of 16:1 and 18:1, respectively, as compared to milk TG in this study (Fig. 3). MUFA 20:1 and 22:1, which are found at unusually high levels in some marine oils and milks (*e.g.*, Fig. 3), were virtually absent from the *sn*-2 position of milks of all marine species studied (Fig. 2 and Table 2) and thus primarily at the α-position (Fig. 5). In human milk, 20:1 has been reported to be preferentially located at *sn*-1 (7); locations of 20:1 and 22:1 have not been reported for the milks of other species. Reasonably high levels of release during gastric hydrolysis, yet at slightly reduced levels in FFA compared to TG (Fig. 3), suggest their tendency also to be esterified to the *sn*-1 position in seal milk.

While the *sn*-2 position of pinniped milk TG was found to contain variable and sometimes relatively high proportions of the minor PUFA 18:2n-6, 18:3n-3, 18:4n-3 and 20:4n-6 (Table 2), these fatty acids usually accounted for less than 1% of fatty acids. Data from other species indicate variable distribution of 18:2n-6 and 18:3n-3, but the majority of both tend to be found at the *sn*-3 position (63). The most important long-chain n-3 PUFA of seal milk, comprising up to 26% of all fatty acids (28), are 20:5n-3, 22:5n-3 and 22:6n-3. These fatty acids were found to be essentially absent from the *sn*-2 position in all species (Fig. 2 and Table 2) and are most likely located primarily at the *sn*-3 position (40,63). The slightly higher levels of these PUFA reported at the *sn*-2 position in the milks of the fur seal, elephant seal and fin whale (64,65) may have resulted from the use of pancreatic lipase rather than Grignard hydrolysis for positional analysis. Thus, the fatty acids most preferentially released from the milk TG during gastric digestion (other than 16:1 and 18:0) were the PUFA of chain-length 18 carbons or longer (Fig. 3), which is consistent with their location in the milk TG (*e.g.*, Fig. 5) and the specificity of gastric lipase for the *sn*-3 position. These findings were supported by the relative depletion of the long-chain PUFA in the DG fraction (Fig. 4). Preferential release during intragastric hydrolysis has also been reported in puppies for the major PUFA of dog milk, 18:2n-6, 18:3n-3 and 20:4n-6 (47).

The single PUFA which did not fit this pattern was 22:6n-3. While the extraordinary enrichment of 20:5n-3 in FFA during gastric hydrolysis was surprising (Figs. 3 and 4), the extreme reduction of 22:6n-3 in FFA was even more surprising since it is likely that 22:6n-3 also occurs

TABLE 2

Comparison of Percentage Distribution of Fatty Acids at the *sn*-2 Position in Milk TG of Various Marine Mammals[a]

	14:0	16:0	18:0	16:1	18:1	20:1	22:1	18:2 n-6	18:3 n-3	18:4 n-3	20:4 n-6	20:5 n-3	22:5 n-3	22:6 n-3
Range in milks[b]	2–11	9–23	1–4	4–17	19–39	2–17	0.3–7	1–2	0.3–1	0–1	0.3–1	1–14	1–4	4–16
CA sea lion[c]	100	90	0	50	0	0	0	48	52	62	0	4	4	0
Antarctic fur seal[d]	63	79	0	37	13	0	0	—	—	—	—	7	0	0
Harp seal[c]	76	79	17	60	30	0	0	59	44	64	11	6	5	0
Hooded seal[c]	79	92	74	38	27	3	1	49	6	0	0	0	0	0
N. elephant seal[d]	78	92	20	57	26	6	7	—	—	—	—	13	3	16
Weddell seal[e]	68	69	11	41	16	—	—	37	33	----------2----------				
Fin whale[f]	84	80	7	57	12	2	1	18	31	45	11	8	4	6

[a] Percent of each fatty acid present in milk which is located at the *sn*-2 position of the triglyceride (TG) as determined from positional analyses; dashes indicate values not reported or analyzed. % @ *sn*-2 = [*sn*-2]/3[TG] × 100. CA, California.

[b] Total weight % present in milk TG of these species (ref. 28, this study).

[c] From this study.

[d] Calculated from ref. 64.

[e] Calculated from ref. 63. All PUFA > 18:3 reported as a single value.

[f] Calculated from ref. 65.

primarily at the *sn*-3 position (this study, refs. 40 and 63). Additionally, like 20:5n-3 and 22:5n-3, proportions of 22:6n-3 were found to be depleted in the gastric DG obtained from all three species, suggesting that it also had been released from the TG (Fig. 4). In all samples, 22:6n-3 was stable in all fractions (total, TG, DG, FFA) and was readily and reproducibly collected from preparative plates, transesterified and measured. The lower levels of 22:6n-3 found in both the DG fraction (as expected) and FFA fraction were considered to be accurate measurements; no evidence of loss through autooxidation or overlap in various fractions was found, despite numerous efforts to elucidate such occurrences. The DG and FFA data, in addition to the residual TG available, also demonstrated no evidence of intermolecular specificity by lipases (*i.e,* preferential hydrolysis of TG species). Thus the extreme reduction of 22:6n-3 in FFA, in conjunction with the enrichment of 20:5n-3 in FFA at levels much higher than expected, suggests some kind of metabolic loss, conversion or preferential absorption. The retroconversion of 22:6n-3 to 20:5n-3 has previously been reported (67–69), although how this process could possibly occur during digestive metabolism and whether this could account for the observed discrepancies is unclear. Given that most hydrolyzed lipids are believed to remain with the parent milk fat globule until its disruption in the intestine (23,24), preferential absorption of a single and very long-chain fatty acid, such as 22:6n-3, seems unlikely. Clearly, these patterns require further investigation.

The rapidly accumulating information on the effects of n-3 PUFA in marine oils confirms the notion that these are important dietary components for both infants and adults (4–7). Nevertheless, this stands in contrast to the relatively limited knowledge about the digestion of n-3 PUFA, particularly from milk TG. In this study, high intakes of total milk fat and PUFA were coupled with high levels of gastric lipolysis in pups. Despite apparent individual and species variation in the extent of hydrolysis, the same fatty acids, primarily the long-chain and n-3 PUFA, were preferentially released in pups of all three species. This is explained by their unique location in the milk TG at the α- and probably mostly at the *sn*-3 position and by the corresponding specificity of gastric lipase. Reports of preferential hydrolysis of short- and medium-chain fatty acids in milk (*e.g.*, ref. 66) may be partly attributable to the fact that these fatty acids, at least in the human and the rat, are also preferentially esterified at the *sn*-3 position in milk (70,71).

We conclude that the n-3 PUFA in milk are efficiently hydrolyzed by gastric lipase and that this has important implications for the digestion of milks enriched in PUFA by neonates in general. Given the general similarity in milk TG structure among species and the fact that many mammalian species, including the human, possess lingual and/or gastric lipases, hydrolysis of milk fat enriched in the important long-chain n-3 PUFA may be primarily dependent upon the actions of these lipases prior to reaching the intestine. Furthermore, while pancreatic lipase may not be able to continue hydrolysis of these fatty acids in the α-positions, bile salt-stimulated lipase, present in the milk of species such as seals, humans and a number of other primates and carnivores studied, non-specifically hydrolyzes TG and partial glycerides in the intestine (72–74), and may account for further PUFA release.

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n-3 Fatty Acid Requirements of the Newborn[1]

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Whether docosahexaenoic acid (22:6n-3) is an essential nutrient for term or preterm infants, or if not, the quantity of dietary linolenic acid (18:3n-3) needed to support sufficient synthesis of 22:6n-3 for assimilation in the central nervous system is unknown. Infants fed formulas have lower plasma and red blood cell (RBC) levels of 22:6n-3 than breast fed infants. No relationship between the intake of 18:3n-3 in formula (0.8 or 4.5% of fatty acids, 18:2n-6/18:3n-3 ratio 35:1 or 7:1, respectively) and the infant's RBC 22:6n-3 was found. Premature infants (<33 wk gestation) also showed a decrease in RBC 22:6n-3 during feeding with formula containing 18:3n-3 as the only n-3 fatty acid. However, a marked decrease in plasma and RBC 22:6n-3 occurred between premature birth and the start of full enteral feeding at 1-2 wk of age. This was not reversed by breast milk or formula feeding. Piglets, which are appropriate for studies of infant lipid metabolism, had decreased brain synaptic plasma membrane, retina and liver 22:6n-3 and increased 22:5n-6 when fed formula with 0.8% fatty acids (0.3% of kcal) as 18:3n-3. Formula with 4.0% fatty acids (1.7% of kcal) as 18:3n-3 resulted in similar accretion of 22:6n-3 in the organs compared to milk fed animals. The studies suggest the dietary requirement for 18:3n-3 in term animals in energy balance exceeds 0.3% diet kcal. Studies in the premature infants suggest 18:3n-3 may be oxidized rather than desaturated to 22:6n-3 if energy requirements are not met, and that due to early lipid restriction and later rapid growth, premature infants may have higher dietary n-3 requirements than term infants.
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The two fatty acids accepted as essential dietary nutrients for humans are linoleic acid (18:2n-6) and linolenic acid (18:3n-3) (1). Once obtained from the diet, 18:2n-6 and 18:3n-3 may undergo sequential desaturation and elongation to a series of longer chain polyunsaturated fatty acids (LCP) with 20 or 22 carbon atoms and 3 to 6 double bonds. Of these, arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) are of particular interest because they are found in very high concentrations in membrane structural lipids of the central nervous system (CNS), especially the excitable membranes such as those of the synaptic terminals and visual elements of the retina (2,3).

Synthesis of 20:4n-6 depends on sequential desaturation with alternate elongation of 18:2n-6 by Δ6 and Δ5 desaturases. The synthesis of 22:6n-3 from 18:3n-3 involves similar Δ6 and Δ5 desaturation and elongation, to 22:5n-3, then additional elongation and desaturation, possibly by Δ6 desaturase, and β-oxidation (4). Substrate competition between 18:2n-6 and 18:3n-3 for the Δ6 desaturase may influence the synthesis of 20:4n-6 and 22:6n-3; however, clear preference of the enzyme for 18:3n-3 over 18:2n-6 is known (5-7). The desaturase enzymes are also sensitive to inhibition by the LCP products of either the n-6 or n-3 fatty acid pathway (5,8,9). Consequently, the quantity and balance of 18:2n-6 and 18:3n-3 in infant diets, as well as the addition of n-3 LCP without 20:4n-6, has important implications for the synthesis of 20:4n-6 and 22:6n-3 in developing tissues (1). In addition to the diet 18:2n-6 and 18:3n-3 supply, the fatty acid desaturase enzyme mass and activity, and utilization of fatty acids for energy, can also be expected to influence the rate of LCP formation from 18:2n-6 and 18:3n-3.

It has been known for many years that dietary deficiency of 18:2n-6 and/or 18:3n-3 during development may have long-term consequences with regard to CNS function (10-18). The characteristic fatty acid compositional changes which occur in brain and other organs during essential fatty acid deficiency (deficiency of both 18:2n-6 and 18:3n-3) are an increase in 20:3n-9/20:4n-6 (triene/tetraene) ratio, but maintenance of the normal 20:4n-6/22:6n-3 ratio (1). Dietary deficiency of only 18:3n-3 results in decreased tissue lipid 22:6n-3, as well as increased 20:4n-6/22:6n-3, but not 20:3n-9. Levels of 22:5n-6 seem to be increased in CNS tissues of animals fed diets deficient in both 18:2n-6 and 18:3n-3 or diets deficient in only 18:3n-3 (1). Abnormalities in visual function assessed by electroretinograph (ERG) and direct measures of visual performance (11,15,16) and altered learning (11,17,18) have been found in animals fed diets containing less than about 0.015% kcal 18:3n-3. The functional deficit is presumably related to the accompanying reduction in CNS 22:6n-3. Evidence of reduced visual acuity also has been described in premature infants with low circulating lipid levels of 22:6n-3 (19,20). The functional roles of the normally high levels of 20:4n-6 in the CNS (3), beyond the role as precursor for synthesis of eicosanoids (21), are not well understood.

Large amounts of 20:4n-6, 22:4n-6 and 22:6n-3 are deposited in the CNS during the rapid phase of brain growth (22-25) known as the brain growth spurt (26). In human this commences around the beginning of the third trimester of gestation and continues through the first 18 mon after birth (26). The n-6 and n-3 LCP in fetal and infant tissues are probably derived, at least in part, by placental transfer before birth and from the milk fat after birth. These preformed n-6 and n-3 LCP are undoubtedly taken up and incorporated into developing organ structural lipids (1). The extent to which the developing fetus or newborn depends on a preformed source of 20:4n-6 and 22:6n-3, however, or the amounts of 18:2n-6 and 18:3n-3 needed to achieve adequate synthesis of 20:4n-6 and 22:6n-3 to support optimal composition and function in the developing CNS lipids, is not yet clear.

Our laboratory has combined clinical studies of plasma and red blood cell (RBC) phospholipid fatty acids in term and in preterm infants (1,27-30), with detailed tissue compositional studies of piglets fed similar defined formulas or natural milk (31-36). The parallel studies in the piglet

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Abbreviations: CNS, central nervous system; ERG, electroetinograph; LCP, long-chain polyunsaturated fatty acids of carbon chain ≥ 20 and containing > 2 double bonds; LDL, low density lipoprotein; PC, choline phospholipids; PE, ethanolamine phospholipids; RBC, red blood cell(s); UI, unsaturation index.

have addressed concerns over specificity and sensitivity of plasma and RBC fatty acids, which are influenced by the composition of the fat absorbed from the diet (1), as measures of the n-6 and n-3 LCP composition in developing organs of formula or natural, milk-fed animals. The studies with piglets have included comparison of the efficacy of dietary 18:3n-3 as compared to 22:6n-3 as a source of 22:6n-3 for developing hepatic and CNS structural lipids (31–33). Similarities in physiology and metabolism, brain lipid and natural milk lipid composition (34–36), and in the timing of the brain growth spurt in relation to birth between the piglet and human (26) combine to make the piglet useful for study of fatty acid requirements as they pertain to the human infant. Results of studies on the effect of premature birth and intravenous nutrition (1,27), and formula or breast milk feeding on the plasma and RBC n-3 fatty acids of preterm (1,27,28) and term (1,29,30) infants are discussed in the present paper. Data on the accretion of n-6 and n-3 LCP in CNS membranes, liver and circulating lipids of piglets fed formulas varying in quantity and balance of 18:2n-6 and 18:3n-3, or with marine oils (31–36) are reviewed with regard to interpretation of the effect of milk and formula feeding on the circulating lipids of infants. All of the methods and results discussed from studies with piglets have been published (see references cited).

MATERIALS AND METHODS

Studies of term gestation infants fed breast milk or formulas varying in 18:3n-3. Term gestation infants were fed formulas containing (in % fatty acids) about 32% 18:2n-6 and 4.5% 18:3n-3 (18:2n-6 to 18:3n-3 ratio about 7:1), or 28% 18:2n-6 and 0.8% 18:3n-3 (18:2n-6 to 18:3n-3 ratio about 35:1), or were breast fed from <3 days to 8 wk after birth (n = 16 infants per group). Blood samples were taken by venipuncture at 3 d, and 4 and 8 wk of age, and the plasma and RBC separated. Plasma phospholipids were separated from other lipid classes, and RBC choline phospholipid (PC) and ethanolamine phospholipid (PE) were separated by thin-layer chromatography. The fatty acid composition of each fraction was analyzed by capillary column chromatography. Plasma cholesterol concentration was determined enzymatically. All of the methods have been published in detail (31–36).

Studies of premature infants fed formula or breast milk. The effect of feeding a preterm formula containing (in % fatty acids) about 20% 18:2n-6 and 2% 18:3n-3 or the mother's expressed breast milk on the fatty acid composition of plasma phospholipid and RBC PC and PE of very premature infants was studied using a longitudinal, repeated measures design (28). The expressed breast milk, based on analyses of the milk fed to infants, had (mean wt% fatty acids ± SEM) 11.1 ± 0.8% 18:2n-6, 0.9 ± 0.2% 18:3n-3, 0.8 ± 0.1% 20:4n-6 and 0.5 ± 0.1% 22:6n-3 with about 1.1% total n-6 LCP and 0.9% total n-3 LCP. The infants were of appropriate weight for gestational age, growing well and had an enteral intake of ≥120 kcal/kg/d with no intravenous nutrient support at the start of the study. Blood samples were obtained by venipuncture at enrollment into the study and again 28 d later. Plasma phospholipid, RBC PC and PE fatty acids, and the plasma cholesterol concentration were determined. Characteristics of the infants, including postnatal age, intravenous nutrition from birth to full enteral feeding, and growth during the study have been published (28).

Importance of gestational age at birth and subsequent parenteral nutrition on circulating lipid n-3 fatty acids. Cord blood was collected from fetuses or infants delivered at 20–41 wk post conception (n = 55) to allow study of the potential changes in the plasma and RBC n-6 and n-3 fatty acids with increasing gestational age. Blood was collected from the clamped cord within 5 min of delivery.

Changes in the plasma phospholipid and RBC PC and PE fatty acid composition of premature infants, less than 33 wk gestation and of appropriate weight for gestational age, were studied longitudinally from birth through parenteral nutrition support until full enteral feeding was attained. Intravenous and enteral nutrition was at the discretion of the attending physicians and not altered in any way because of this study. At 72 h post-conception, infants had received parenteral dextrose and amino acids, but no intravenous lipid, with or without enteral feeds with formula (containing in fatty acids, 20% 18:2n-6, 2% 18:3n-3). Analysis of the plasma phospholipid fatty acids from these infants was used to study the effect of 72 h dietary deficiency of n-6 and n-3 fatty acids (23).

Ethical approval for all studies involving infants and cord blood was obtained from the University of British Columbia, British Columbia Childrens' Hospital and Grace Hospital's Clinical Screening Committees for Research and Other Studies Involving Human Subjects (Vancouver, Canada). Written informed consent from a parent was obtained in all cases.

RESULTS

Studies of term gestation infants fed breast milk or formulas varying in 18:3n-3. The fatty acid composition of the plasma phospholipid and RBC PC and PE of the term gestation infants fed the formulas varying in 18:3n-3 content or breast fed has been reported (1,29). For the present purpose, the plasma phospholipid and RBC PE levels (wt% total fatty acids) of 20:4n-6, 22:4n-6, 22:5n-6, 22:6n-3 and unsaturation index (UI) at 3 d, 4 and 8 wk of age are summarized in Figures 1 and 2. Although several consistent and marked differences were found in the plasma phospholipid and RBC PE, as well as in PC, n-6 and n-3 fatty acids levels between the breast and formula fed infants, no differences were found in these fatty acids between infants fed the formula with 0.8% 18:3n-3 and those fed the formula with 4.5% 18:3n-3. The plasma phospholipid % 18:2n-6 were significantly higher in three-day-old infants fed formula than in the breast fed infants (Fig. 1). This difference persisted throughout the 8-wk feeding study. The plasma phospholipid % 20:4n-6 and 22:6n-3 and the (UI) were significantly lower in the formula compared to breast fed infants at both 4 and 8 wk of age.

The RBC PE is distinctly different from plasma phospholipid and is known to contain much lower levels of 18:2n-6 and higher levels of 20:4n-6, 22:4n-6 and 22:6n-3. The CNS also has a very low content of 18:2n-6, usually 1–2% fatty acids, and large amounts of 20:4n-6, 22:4n-6 and 22:6n-3 (3). The effects of milk and formula fats on the fatty acid composition of RBC PE are of interest, because it resembles CNS lipids more closely than RBC PC or plasma lipids, and because it is readily accessible in clinical studies with infants. As found in plasma

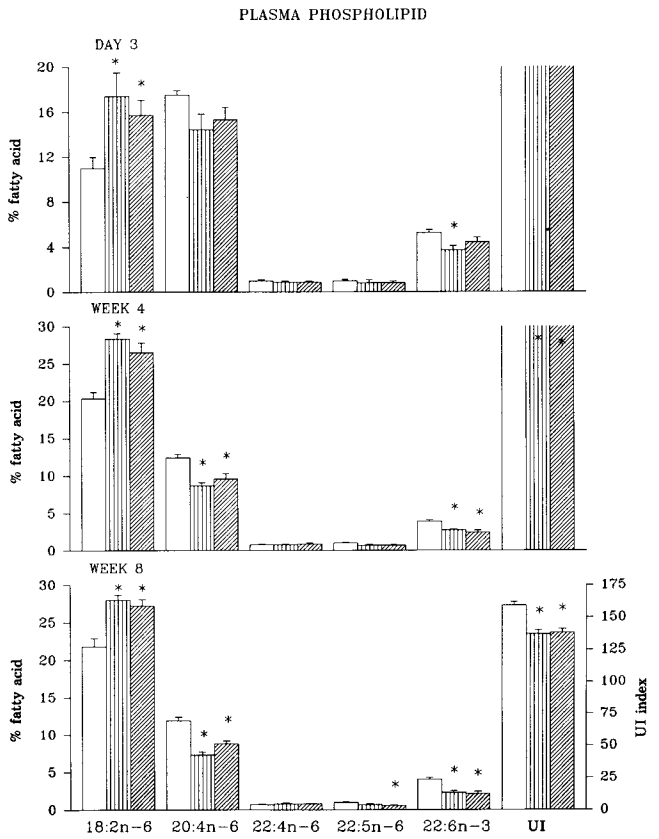

FIG. 1. Major n-6 and n-3 fatty acids (wt% total) and unsaturation index (UI) in plasma phospholipids of term gestation infants who were breast fed or fed formula differing in 18:3n-3 from <3 d to 8 wk of age. Values given are means ± SEM for breast fed infants (open bars) and infants fed the formula containing (in fatty acids) 27% 18:2n-6, 4.5% 18:3n-3 (vertical hatch), or 30% 18:2n-6, 0.8% 18:3n-3 (diagonal hatch). Significant differences ($P < 0.05$) between infants fed formula as compared to breast milk are indicated by an asterisk. There were no significant differences as a result of the type of formula fed. The UI index was calculated as the sum of the wt% fatty acid × number double bonds as in ref. 36.

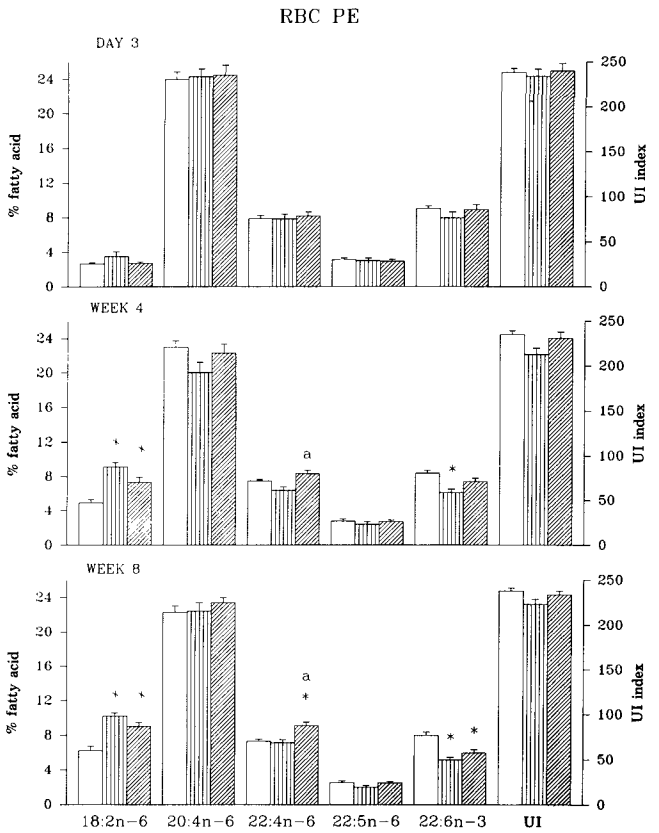

FIG. 2. Major n-6 and n-3 fatty acids (wt% total) and unsaturation index (UI) in red blood cell (RBC) ethanolamine phospholipids (PE) of term gestation infants who were breast fed or fed formula differing in 18:3n-3 from <3 d to 8 wk of age. Values given are means ± SEM for breast fed infants (open bars), and infants fed the formula containing (in fatty acids) 27% 18:2n-6, 4.5% 18:3n-3 (vertical hatch), or 30% 18:2n-6, 0.8% 18:3n-3 (diagonal hatch). Significant differences ($P < 0.05$) between infants fed formula as compared to breast milk are indicated by an asterisk, and infants fed the formula with 0.8% 18:3n-3 compared to those fed the formula with 4.5% 18:3n-3 are indicated by the letter a. The UI index was calculated as the sum of the wt% fatty acid × number double bonds as in ref. 36.

phospholipid, the RBC PE % 18:2n-6 was significantly higher and 20:4n-6 was significantly lower in infants fed the formulas as compared to breast milk for 8 wk (Fig. 2). There were no differences in RBC PE % 22:6n-3 in infants fed formula with 0.8 compared to 4.5% fatty acids as 18:3n-3. The RBC PE % 20:4n-6, and all other C_{22} n-6 and n-3 fatty acids, and the UI also were similar between the breast and formula fed infants with the single exception that the % 22:4n-6 was significantly higher in eight-week-old infants fed the formula with 0.8% 18:3n-3 than in the breast fed infants.

The plasma cholesterol and triglyceride concentrations were significantly higher after 4 wk ($P < 0.01$) and 8 wk ($P < 0.001$) in the breast fed compared to formula fed infants. There were no significant differences in the plasma cholesterol and triglyceride concentration between the two groups of formula fed infants. Results for the plasma cholesterol concentrations at 8 wk of age were (mean ± SEM) 2.93 ± 0.2, 2.91 ± 0.15 and 4.98 ± 0.36 mmol for infants fed the formula with 4.5% 18:3n-3, the formula with 0.8% 18:3n-3 or breast fed, respectively. The triglyceride concentrations in the same three groups at 8 wk of age were (mean ± SEM) 1.70 ± 0.21, 1.41 ± 0.09 and 2.53 ± 0.19 mmol, respectively.

Studies of premature infants fed formula or breast milk. Infants who were fed their mother's expressed breast milk (n = 9) or formula (n = 16) were 12.4 ± 1.4 or 13.9 ± 2.1 days old, respectively, when full enteral feeds of at least 120 kcal/kg/day were attained. The plasma cholesterol decreased significantly, from 3.41 ± 0.29 to 2.58 ± 0.28 mmol and from 4.55 ± 0.44 to 3.05 ± 0.28 mmol over the following 28 d of feeding with the mother's expressed breast milk or formula, respectively. The plasma cholesterol concentration in the infants fed formula and infants fed breast milk were not significantly different at either time point. The concentration of cholesterol in cord blood was 2.46 ± 0.13 mmol for infants of similar prematurity (24) and 1.63 ± 0.21 mmol for term gestation infants (Innis, S.M., and Hamilton, J.J., unpublished data).

No significant differences were found in the % n-6 and n-3 LCP composition of the RBC PE, PC or plasma phospholipid between infants fed expressed breast milk

and infants fed the formula with 20% 18:2n-6, 2% 18:3n-3 for 28 d (25). Repeated measures of longitudinal analysis of variance, however, showed a significant decrease in the RBC PE % 22:6n-3 during the feeding study in infants fed the formula, but not in those fed breast milk (Fig. 3).

Effect of gestational age at birth and subsequent parenteral nutrition on circulating lipid n-3 fatty acids. The % 22:6n-3 in RBC PC, PE and plasma phospholipid was lower in premature infants at the start of full enteral feeding, and after 4 wk feeding with expressed breast milk or formula, than found in newborn or 4-wk-old term gestation infants fed breast milk (37). It seemed important, therefore, to determine whether this was due to premature birth or had occurred during the initial two weeks of parenteral nutrition prior to establishing full volume enteral feeding. Regression analysis of the RBC PC and PE % 22:6n-3 from preterm and term infant cord blood against gestational age showed either no change or a positive correlation with increasing gestation from 20 to 41 wk (1; Fig. 3). Levels of 22:6n-3 decreased, however, from birth to the start of full enteral feeding. Subsequent feeding with either breast milk or formula did not restore the RBC PE 22:6n-3 to the levels of about 8-9% fatty acids found at birth and in cord blood of newborn infants of equivalent post-conceptional age (Fig. 3).

Premature infants (26–34 wk gestation) maintained for the first 72 h after birth with parenteral dextrose and amino acid solutions and no exogenous lipid had higher % and mg/dL 18:1 and 20:3n-9 in their plasma phospholipid than found in cord blood or in plasma phospholipid of similar 72-h-old premature infants given formula (Fig. 4; ref. 27). The elevation of 18:1 and 20:3n-9 provides biochemical evidence of rapidly developing essential fatty acid deficiency. The mg/dL 20:4n-6, 22:4n-6, 22:5n-6, 22:5n-3 and 22:6n-3 in plasma phospholipids also increased significantly in infants deprived of lipid for the first 72 h after birth. It is reasonable to assume that this increase in plasma LCP was due to mobilization of tissue fatty acids, and secretion as hepatic lipoprotein phospholipid. The exogenous energy supply in all the infants at this time was less than the maintenance requirements for premature infants in a thermoneutral environment, usually assumed to be at least 50–60 kcal/kg/day (38).

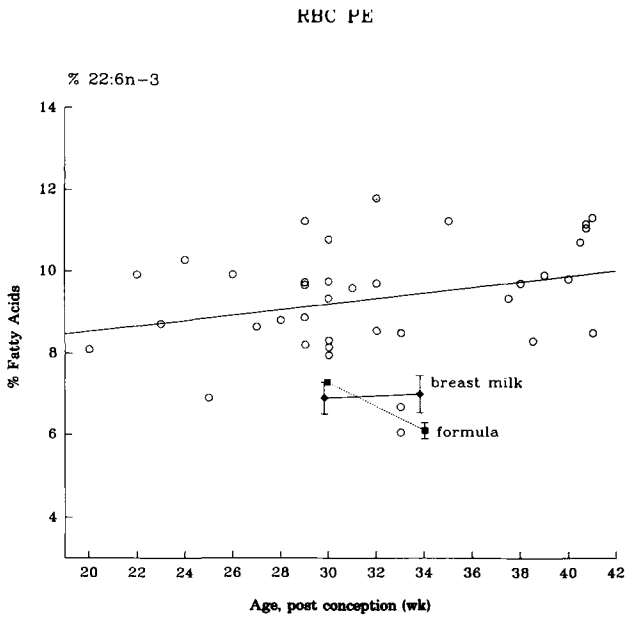

FIG. 3. Content of 22:6n-3 (% total fatty acids) in the red blood cell ethanolamine phospholipids (RBC PE) of cord blood and of premature infants fed breast milk or formula. The premature infants were fed their own mother's expressed breast milk or formula (with 2% fatty acids as 18:3n-3) for four weeks, following birth at 29.4 ± 0.4 and 28.2 ± 0.6 weeks postconception, respectively. The % 22:6n-3 in the RBC PE decreased in infants fed the formula ($P < 0.05$), but was not significantly different from infants fed expressed breast milk at any time in the study.

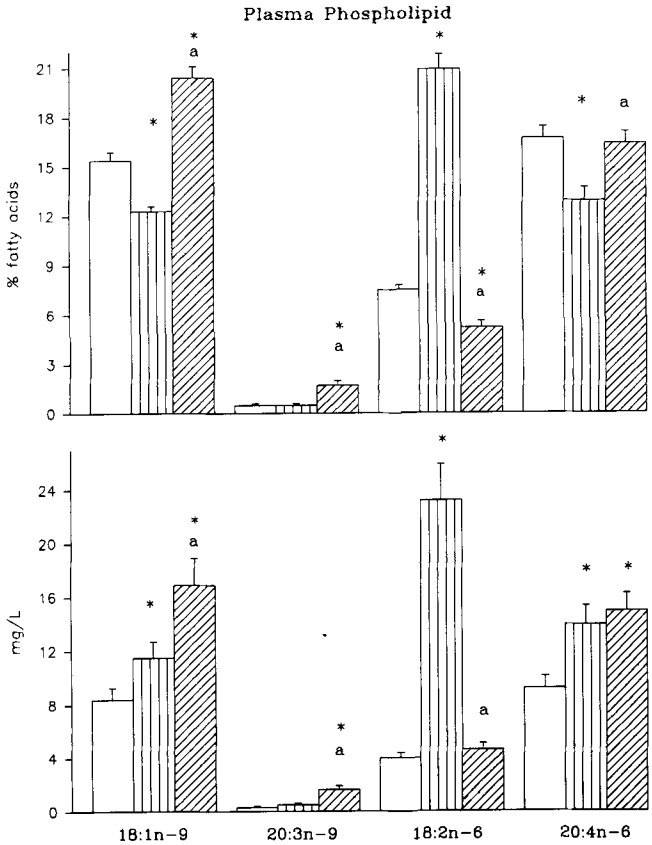

FIG. 4. Plasma phospholipid n-9 and n-6 fatty acids (% total and mg/dL) of premature infants at birth, and at 3 d of age. Values given are means ± SEM for cord blood (n = 22, gestation 29.5 ± 2.3 wk, open bars) and 72-h-old infants who received intravenous dextrose and amino acids with enteral feeds of formula (140–297 mL/kg total, vertical hatch); (n = 12, gestation 31.8 ± 1.4 wk) or no enteral feeds (n = 15, gestation 28.1 ± 2.0 wk). Significant differences ($P < 0.05$) between the 72-h-old infants and the cord blood are indicated by an asterisk, and between the infants who received no lipid compared to those fed formula are indicated by the letter a.

DISCUSSION

The results of analyses presented here showing lower % 22:6n-3 in plasma and RBC phospholipids of term gestation infants fed formulas containing blends of vegetable oils with no n-6 or n-3 LCP, rather than breast milk, are similar to the results of others (1,39). Premature infants fed formulas, but not those fed expressed breast milk, also show a significant decline in their RBC PE % 22:6n-3

(Fig. 3). The difference in the levels of 22:6n-3 between the premature infants fed formula and those fed breast milk, however, did not reach statistical significance in this 28-d study. Similar levels of 22:6n-3 in RBC lipids of preterm infants fed formula containing 2.7% total fatty acids as 18:3n-3 and preterm infants fed breast milk from less than 11 d to 25–36 d of age have been found by others (20). Studies using a longer feeding period of 47 d, however, found significantly lower RBC PE % 22:6n-3 in premature infants fed formulas with 0.6–0.9% 18:3n-3 rather than breast milk (36).

It seems clear from clinical studies with term and preterm infants fed formulas that either: (i) dietary 18:3n-3 is not desaturated to 22:6n-3; or (ii) 18:3n-3 is desaturated to 22:6n-3 but the 22:6n-3 formed in the organs is not secreted into plasma in amounts related to the diet 18:3n-3 supply, or to equal the levels circulating when 22:6n-3 is absorbed from breast milk lipid. Failure to achieve adequate desaturation of 18:3n-3 could be explained by an inadequate dietary supply of 18:3n-3, competitive inhibition of 18:3n-3 desaturation by the high 18:2n-6 in the formulas, or limited capacity for desaturation-elongation of 18:3n-3 to its LCP products.

Substrate competition between 18:2n-6 and 18:3n-3 for Δ6 desaturation *in vitro* and *in vivo* in animals fed diets limiting in 18:3n-3 or with very high 18:2n-6 is well known (5–7). The similar circulating lipid levels of 22:6n-3 in term gestation infants fed formulas containing 27–30% 18:2n-6 with 0.8 or 4.5% fatty acids as 18:3n-3 (Figs. 1 and 2) suggest that ratios of 18:2n-6 to 18:3n-3 over the range of 7:1 to 35:1 were not a determinant of the circulating lipid 22:6n-3 in the infants fed formula in these studies.

Relatively little attention has been given to the marked differences in n-6 fatty acids between infants fed breast milk and infants fed formula. The significantly higher levels of 18:2n-6 in the plasma phospholipids of term and preterm infants fed formula as compared to those fed breast milk (28,29,39,40) may reasonably be related to the higher levels of 18:2n-6 in many formulas than in typical breast milks (41). The reason for the lower 20:4n-6 in association with higher 18:2n-6 in the plasma phospholipids of the infants fed formulas, however, is not known. The results suggest inability of, or interference with, desaturation of 18:2n-6. This could be due to low Δ6 and/or Δ5 desaturase activity in newborn infants, or possible substrate inhibition of desaturation (1) by the relatively high (about 12–15% kcal) 18:2n-6 in the formulas. Either explanation could provide a common reason for the accompanying decrease in plasma and RBC phospholipid levels of 22:6n-3. The RBC PE % 20:4n-6 is much higher than that found in plasma phospholipids. Despite this, the decreased plasma phospholipid 20:4n-6 associated with formula feeding (Fig. 1) was not associated with reduced levels of 20:4n-6 in the RBC PE (Fig. 2). This, together with the decrease in plasma phospholipid 20:4n-6 with increasing age in all infants, irrespective of feeding group, suggests that factors other than desaturase enzyme activity may be important to the metabolism of plasma n-6 fatty acids.

As has been reported by others (42), the plasma cholesterol concentration was significantly lower in term gestation infants fed formula than in breast fed infants. Infant formulas usually supply about 4 mg cholesterol/dL, whereas analyses of mature human breast milk typically report cholesterol concentrations of 20–50 mg/dL (41). The esterification of plasma-free cholesterol by lecithin:cholesterol acyltransferase involves transfer of 18:2n-6 from the *sn*-2 position of plasma PC to free cholesterol (43). It seems reasonable to question whether or not the lower dietary cholesterol intake and plasma cholesterol concentration in formula fed infants results in slower turnover of plasma phospholipid 18:2n-6 than in breast fed infants. The higher plasma cholesterol concentration in preterm than term infants, and the decline in plasma cholesterol in preterm, compared to increase in term infants, over the first month of full enteral feeding (44) provides evidence of marked differences in plasma cholesterol and lipoprotein metabolism. Whether or not this has implications for the metabolism of lipoprotein phospholipid 18:2n-6, or other n-6 or n-3 fatty acids, has yet to be considered.

An increase in tissue lipid n-6 fatty acids, particularly 22:5n-6, is a characteristic feature of nonhuman primates, rodents and piglets fed diets deficient in 18:3n-3 (1,10, 15–18,31–36). The lack of a consistent increase in plasma and RBC PC or PE 22:5n-6 in the term gestation infants fed the formulas with 0.8 or 4.5% fatty acids as 18:3n-3 (Figs. 1 and 2), or in preterm infants fed breast milks or formulas (28) could be interpreted as evidence that there was no overt biochemical deficiency of n-3 fatty acids in the infant tissues. The ability to respond to n-3 fatty acid deficiency with an increase in 22:5n-6, however, is dependent on desaturation of 18:2n-6 in liver, and subsequent secretion of the n-6 LCP products into plasma as lipoprotein phospholipid. Thus, until optimal activities of the desaturase enzymes in infant tissues are assured, and it is known that 22:5n-6 is incorporated into secreted lipoprotein phospholipid, findings of "low" C_{22} n-6 LCP in the circulating lipids of infants should not be considered as evidence of adequate tissue levels of 22:6n-3.

Studies in nonhuman primates and rodents have shown biochemical and functional deficiency of 22:6n-3 as a result of feeding diets with less than 0.02% kcal 18:3n-3 (1,10, 15,16). The control diets in the latter studies provided >0.3% kcal 18:3n-3 (usually from soybean oil), but no LCP. Biochemical deficiency, based on increased 22:5n-6 and decreased 22:6n-3 in brain, its synaptic plasma membranes, retina and liver has been shown in piglets fed exclusively from birth with formula containing 0.8% fatty acids (0.35% kcal) as 18:3n-3 rather than sow milk (34–36). Liver and CNS levels of 22:6n-3 were similar or higher in piglets fed formula with 4% fatty acids (1.7% of kcal) as 18:3n-3 to that in piglets fed sow milk (31). The increase in 22:5n-6 in developing rodents, piglets and nonhuman primates fed diets limiting in 18:3n-3, and the accumulation of large amounts of 22:6n-3 when generous amounts of 18:3n-3, but no LCP, are fed (10,15–18,31–36) provides clear evidence of adequate desaturase activity in these term newborns. The results from studies on the CNS synaptic plasma membranes and retina PE, which usually contain very high levels of 22:6n-3, show that when using the amounts and ratios of 22:5n-6 and 22:6n-3 in these tissues as criteria of dietary n-3 fatty acid adequacy, the dietary requirement for n-3 fatty acids in the exclusively formula-fed term gestation piglet is between 0.35 and 1.7% of diet kcal.

Small amounts of fish oil, to provide up to 1% fatty acids (0.5% kcal) in formula as n-3 LCP (2 to 6 g menhaden oil/L formula), also have been shown to be effective in

supporting similar levels of 22:6n-3 in CNS lipids of piglets to levels found in naturally suckled piglets (31-33). The quantitative superiority of dietary 20:5n-3 and 22:6n-3, compared to 18:3n-3, as a source of 22:6n-3 for developing membrane tissues is clear. This may be explained by the known differences in affinities for 18:3n-3 and 22:6n-3 of the acyltransferases involved in structural lipid synthesis and turnover, and predominant mitochondrial oxidation of 18:3n-3, but not 22:6n-3 (1). Piglets fed formulas with 20:5n-3 and 22:6n-3 (from marine oil), in contrast to piglets fed 18:3n-3 (from soy or canola oils), showed a significant reduction of 20:4n-6 in liver and plasma phospholipids (32,33). Evidence is available to suggest this may be explained by inhibition of 18:2n-6 desaturation, as well as competition between 20:5n-3 and 20:4n-6 for acylation (1). Although natural milks contain 22:6n-3, levels of 20:5n-3 are normally very low, and 20:4n-6 is always present (41). Although marine oils with 20:5n-3 and 22:6n-3 appear to provide an efficacious dietary source of 22:6n-3 for the developing CNS (31-33), the studies with piglets suggest that they should not be added to infant diets unless a source of 20:4n-6 also is included.

The specificity of measures of plasma and RBC 22:6n-3 in infants fed diets which do (breast milks, marine oils) and do not (vegetable oil formulas) contribute n-3 LCP to the circulating lipid pool, as an index of adequate 22:6n-3 in developing organs, remains an important question. Numerous studies have shown that fish oil supplements increase plasma and RBC 20:5n-3 and 22:6n-3 (1). Levels of 22:6n-3 were also higher in the breast milk and in the RBC lipids of breast fed infants whose mothers followed omnivorous rather than vegetarian diets (45). Studies with the piglet support others in demonstrating that dietary n-3 fatty acid deficiency leading to tissue 22:6n-3 deficiency is accompanied by low plasma and RBC 22:6n-3 (1). The results of studies with these animals, however, also show that diet-related differences in neither plasma nor RBC 20:4n-6 or 22:6n-3 necessarily predict differences in brain or retina in animals fed diets adequate in 18:3n-3 or containing n-3 LCP.

The decrease in n-6 and n-3 LCP in premature infants between birth and the start of breast milk or formula feeding has been recognized (1,37,40). Whether or not the postnatal decrease during parenteral nutrition is due to oxidation of 18:2n-6 and 18:3n-3 for support of vital metabolic functions during periods of inadequate energy intake (1), delay in administration of 18:2n-6 and 18:3n-3 (27), inappropriate content or balance of 18:2n-6 and 18:3n-3 in parenteral lipid emulsions (46,47), or immature desaturase activities in the infant born before term is unclear. It is clear, however, that infants who receive no intravenous lipid, formula or breast milk during the first days after birth (27) will mobilize tissue n-6 and n-3 fatty acids, probably to meet the energy needs of obligatory metabolic and physiological functions. Whether or not this results in subsequent higher n-3 fatty acid requirements may be worth consideration, particularly since the results of studies here have found that neither expressed breast milk nor formulas lacking 22:6n-3 were able to restore circulating 22:6n-3 levels of 2-6-wk-old premature infants to levels found at birth or in cord blood of infants of equivalent post-conceptional age (1).

Results of clinical studies have been presented and show that levels of 22:6n-3 in the plasma and RBC phospholipids of infants change after birth to reflect the differences in the amount of 22:6n-3 in the milk or formula diet. Thus, levels of 22:6n-3 decrease in infants fed formulas, in which the only n-3 fatty acid is 18:3n-3, but remain relatively constant in infants fed breast milk containing 22:6n-3. The decrease in 22:6n-3 of formula fed infants occurs in the order of plasma phospholipid, followed by RBC PC and then the RBC PE, reflecting exchange of the RBC fatty acids with the plasma and the constraints to full equilibration imposed by the specificity of the RBC acyltransferase reactions. Studies in term gestation piglets fed during the usual suckling period with similar formulas to those used in clinical studies have shown adequate fatty acid desaturation-elongation of 18:2n-6 and 18:3n-3 in organs, which cannot be reliably predicted from analysis of circulating lipid fatty acids. The dietary n-3 fatty acid requirement in the piglet for normal assimilation of 22:6n-3 in the brain and retina is >0.3% and <1.7% diet kcal as 18:3n-3. Requirements for n-3 fatty acid can also be met in this species with about 0.3% diet kcal as 20:5n-3 and 22:6n-3, emphasizing the higher biological activity of dietary C_{20} and C_{22} n-3 fatty acids as compared to 18:3n-3.

Results of studies in the preterm infant suggest that dietary 18:3n-3 may be oxidized for energy, and thus not an efficacious precursor for synthesis of 22:6n-3. Reasons for this may include possible low desaturase enzyme activity, early negative energy balance, delayed intravenous lipid administration, subsequent rapid growth and the possible need to restore depleted tissue 22:6n-3 pools. The available information suggests that the n-3 requirements of preterm infants may be higher than for term infants, and may not be adequately met by dietary 18:3n-3.

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Essentiality of Dietary ω3 Fatty Acids for Premature Infants: Plasma and Red Blood Cell Fatty Acid Composition[1]

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Pre-term infants, that are not breast-fed, are deprived of vital intrauterine fat accretion during late pregnancy and must rely on formula to obtain fatty acids essential for normal development, particularly of the visual system. Preterm infants (30 wk postconception) receiving human milk were compared to infants given one of the following formulae: Formula A was a commercial preterm formula with predominantly 18:2ω6 (24.2%) and low (0.5%) 18:3ω3; Formula B was based on soy oil and contained similar 18:2ω6 levels (20%) and high 18:3ω3 (2.7%); Formula C was also a soy oil-based formula (20% 18:2, 1.4% 18:3) but was supplemented with marine oil to provide ω3 long-chain polyunsaturated fatty acids (LCP) at a level (docosahexaenoic acid, DHA, 0.35%) equivalent to human milk. At entry (10 days of age), the fatty acid composition of plasma and red blood cell (RBC) membrane lipids of the formula groups were identical. By 36 wk postconception, the DHA content in lipids of group A was significantly reduced compared to that in the human milk and marine oil formula groups. Omega-3 LCP results were further amplified by 57 wk with compensatory increases in 22:5ω6 in both plasma and RBC lipids. Provision of 2.7% α-linolenic acid in formula group B was sufficient to maintain 22:6ω3 levels equivalent to those in human milk-fed infants at 36 wk but not at 57 wk. Effects on the production of thiobarbituric acid reactive substances and fragility of RBC attributable to the marine oil supplementation were negligible. The results support the essentiality of ω3 fatty acids for preterm infants to obtain fatty acid profiles comparable to infants receiving human milk. Formula for preterm infants should be supplemented with ω3 fatty acids including LCP.
Lipids 27, 886–895 (1992).

Since animals lack the desaturating enzymes (1,2) necessary to form the ω6 and ω3 series of fatty acids (FA), they must rely on plant sources to obtain the parent essential fatty acids (EFA). Animal tissues are capable of further elongating and desaturating the parent EFA, linoleic acid (18:2ω6) and α-linolenic acid (18:3ω3), generating a family of long-chain polyunsaturated fatty acids (LCP) of each respective series. Alternatively, dietary LCP may be provided to infants directly from sources such as breast milk, marine oils or egg phospholipids.

The functional and structural roles of specific long-chain fatty acids are being increasingly recognized. Synthesis of eicosapentaenoic acid (EPA, 20:5ω3) from α-linolenic acid in animals provides a precursor for series-3 prostaglandins which may suppress some effects of the series-2 prostaglandins originating from ω6 FA by limiting their production. Although the actions of EPA may be beneficial in pathological conditions, concern persists as to the safety of limiting the action of arachidonate-derived eicosanoids in infants. Docosahexaenoic acid (DHA, 22:6ω3) may provide a structural micro-environment in the membrane that influences important membrane functions such as ion or solute transport, receptor activity or enzyme action (3,4). The enrichment of DHA in retina and neural tissues (5,6) may reflect a unique and critical role for this ω3 LCP in visual function. Although the essentiality of ω6 FA in the diet has long been recognized, only recently have studies focused on the more subtle biochemical and functional effects of dietary ω3 FA deficiency. The highly unsaturated nature of DHA may be responsible for the observed effects of ω3 FA deficiency on the function of developing brain and retina (3,7). Wheeler *et al.* (8) demonstrated dramatic enhancement of both a- and b-waves of electroretinograms from rats fed dietary supplements of ω3 FA compared to fat-free diets or supplementation with only ω6 FA. Neuringer *et al.* (9) reported visual acuity impairments of up to 50% in newborn rhesus monkeys receiving safflower-based diets deficient in ω3 FA pre- and postnatally compared to controls given a soybean oil-based diet. These primates have depressed levels of DHA in plasma phospholipids at birth and nearly undetectable amounts at 12 wk of age (10). These visual and neural derangements may also occur in human infants; however, evidence of FA status in the brain presently must rely on the direct correlations of brain and red blood cell (RBC) lipid FA distribution found in rat and primate studies (9,11).

During fetal growth in the human, accretion of C_{18}-EFA is not linear but is approximately 400 mg of ω6 and 50 mg of ω3 EFA per kg of body weight deposited daily during the last trimester of gestation (12) and continues postnatally in brain tissue (13). However, a preterm infant born at less than 34 wk will be deprived of this critical period of intrauterine lipid accretion and thus, is dependent on diet to fulfill its EFA requirements. Although formula supplementation with ω6 and ω3 EFA (*i.e.*, 18:2ω6 and 18:3ω3) may be beneficial (14), evidence for a limited FA desaturase activity (15) indicates that LCP such as arachidonic acid (16) and DHA (17,18) may also be required by the low-birth-weight infant.

Preterm infants fed their own mother's milk receive a typical intake of 140 mg ω3 FA/kg/d including 45 mg ω3 LCP/kg/d (19), thus meeting the requirements for neural tissue and whole body EFA accretion. Preterm infants given commercial formula receive less α-linolenic acid and elevated linoleic acid compared to breast-fed infants. This relative excess of 18:2ω6 may limit the formation of ω3

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Abbreviations: ANOVA, analysis of variance, DHA, docosahexaenoic acid (22:6ω3); EFA, essential fatty acid(s); EPA, eicosapentaenoic acid (20:5ω3); FA, fatty acid(s); HM, human milk; LCP, long-chain polyunsaturated fatty acid(s); MCT, medium-chain triglycerides; RBC, red blood cell(s); TBARS, thiobarbituric acid reactive substances.

LCP in cell membranes (20–22) and blood lipids (11,17,18) because the Δ6 desaturase shared by both series of FA is subject to substrate inhibition (1). Typical premature infant formulas available up to 1987 (when the study commenced) had a low content of α-linolenic acid and no ω3 LCP. Thus, we considered the preterm infant at risk for ω3 LCP deficiency because biosynthesis is limited and formula diet supply was insufficient to meet the needs of the developing visual and neural systems (23).

The purpose of the present study was to evaluate the potential effects of ω3 FA deficiency in very-low-birthweight human infants by studying functional and biochemical indicators of ω3 FA status. The FA composition of plasma and RBC lipids of human milk-fed infants and three formula-fed groups receiving different EFA and LCP supplementation are reported herein. A secondary objective included the evaluation of RBC membrane peroxidation and resistance to hemolysis in the four diet groups. Interim visual function results for the infants under study have been recently published (18).

MATERIALS AND METHODS

Subjects. Ninety-five newborns with body weights of 1000–1500 g, who received enteral feedings but were free of major neonatal morbidity by day 10 of life, were eligible for the study. Twelve infants receiving breast milk served as controls for the study. The human milk (HM) fed group (supplemented with Enfamil Fortifier, Mead-Johnson, Evansville, IN) received ⩾75% of their intake as HM up to 36 wk postconceptional age (usual discharge age); if mothers were unable to fully provide HM for a brief period (<48 h), formula C (see below) was used to supplement their feeding. The remaining 83 infants were randomly assigned to three formula groups varying in EFA. Experimental formula feedings began at day 10 of life and continued until 57 wk postconceptional age (*i.e.*, equivalent to 4 mon postterm). Further description of inclusion and exclusion criteria related to the study cohorts have been previously described (18).

Diets. Formula A corresponds to the fat composition of Enfamil Premature (Mead-Johnson Bristol-Myers Co., 1987 formulation) based on a medium-chain triglyceride (MCT)/coconut/corn oil mix and was low in all ω3 FA. Formula B, based on MCT/coconut/soy oil, was adequate in 18:2ω6, enriched in 18:3ω3 but contained no ω3 LCP. Formula C, based on MCT/coconut/soy oil, was enriched with ω3 LCP by addition of marine oil (winterized, deodorized, stabilized menhaden oil provided by Zapata-Haynie Co., Reedville, VA) and was thus comparable in content of ω3 LCP to preterm human milk. After 36 wk, respective full-term formulae of similar EFA composition were given. The diet formulations were specially prepared for this study by Mead Johnson Nutritional Division; the FA compositions are presented in Table 1. The macronutrient composition of formulas A, B and C consisted of protein, 2.4 g/dL; carbohydrate, 8.9 g/dL; and fat, 4.1 g/dL with varying proportions of ω3 FA but similar ω6 FA content. The vitamin and mineral content of the preterm and full-term milk formula met the recommendations of the American Academy of Pediatrics for low-birth-weight neonates and full-term infants, respectively (24). The tocopherol content was at least 2 mg per g unsaturated FA to ensure stability of the LCP in the formula for about 24 mon. FA peroxidation analysis confirmed that this objective was attained.

Fatty acid analysis. The composition of FA in total plasma and total RBC membrane lipids was measured to assess the effect of dietary EFA on biological function and to ensure compliance. FA composition of RBC membrane lipids are correlated with changes in FA of other membranes including brain and retina and have been

TABLE 1

Fatty Acid Composition of Study Diets

	Human milk[a]	Formula A[b]		Formula B[b]		Formula C[b]	
		Preterm	Follow-up	Preterm	Follow-up	Preterm	Follow-up
		(mean values, g/100 g of lipid)					
6:0	—	2.4	0.4	2.5	0.4	2.5	0.4
8:0	1.4	28.3	5.1	28.3	4.9	28.4	5.1
10:0	3.2	10.3	3.9	10.4	3.7	10.4	3.9
12:0	4.7	10.5	30.4	10.5	29.1	10.8	30.6
14:0	8.0	3.4	12.5	3.4	12.4	4.0	13.0
16:0	19.2	6.1	11.0	5.8	10.9	6.4	11.0
18:0	8.9	1.3	3.8	2.1	4.5	1.7	4.4
18:1ω9	35.5	11.8	12.5	10.3	11.9	10.7	11.7
18:2ω6	12.7	24.2	21.1	20.8	20.3	20.4	18.1
18:3ω3	0.8	0.5	0.5	2.7	2.8	1.4	1.4
20:5ω3	0.10	0.0	0.0	0.0	0.0	0.65	0.5
22:6ω3	0.29	0.0	0.0	0.0	0.0	0.35	0.4
Σ ω6 LCP[c]	1.5	0.0	0.1	0.0	0.1	0.1	0.1
Σ ω3 LCP	0.5	0.0	0.0	0.0	0.1	1.0	0.9
Total ω6/ω3	11.4	48.4	45.1	7.7	7.0	8.5	7.9

[a]Representative of day 20 breast milk sample.

[b]Formula A was based on medium-chain triglycerides (MCT)/coconut/corn oil blend, Formula B was based on MCT/coconut/soy oil blend, and Formula C was based on MCT/coconut/soy/marine oil blend. Average of triplicate determinations.

[c]LCP, long-chain polyunsaturated fatty acid(s).

related to functional effects (9,11). In addition, these lipid patterns serve as biochemical indicators of specific dietary FA supply.

Blood samples were obtained by venipuncture from a small arm vein at entry (approximately 32 wk postconception, 10 days postnatally), at 35 to 37 wk (usual discharge age) and at 57 wk postconception. Plasma and RBC were separated by centrifugation (3,000 × *g* for 10 min). RBC were washed twice with saline, and an aliquot was lysed with water prior to lipid extraction using methanol/chloroform as described by Bligh and Dyer (25). Plasma lipids were similarly extracted.

Lipid samples were analyzed immediately or stored briefly under N_2 in solvent containing butylated hydroxytoluene (0.02%) as antioxidant at −20°C until transmethylation. FA of lipids were converted to methyl esters by transesterification under N_2 with 14% boron trifluoride in methanol at 100°C for 10 min (26). FA methyl esters were stored in antioxidant-containing solvent and under N_2 at −20°C until chromatography. FA methyl esters in methylene chloride were separated and quantified using a capillary column gas chromatograph fitted with a flame-ionization detector. Analyses were done on a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) equipped with a 0.25 mm i.d., 30-m capillary column filled with SP-2330 stationary phase (Supelco, Bellefonte, PA). Helium at a flow of 1 mL/min was used as carrier gas; the split ratio was 9:1. The oven temperature was kept at 150°C for 5 min, then raised 4°/min to 220°C, stabilized at 220°C for 8 min, then raised again at 15°/min to 240°C, and finally stabilized at 240°C for 8 min. Peaks were identified by comparison with individual purified standards and standard FA methyl ester mixtures (GLC 68, PUFA2, Rapeseed oil) obtained from either NuChek Prep (Elysian, MN) or Supelco. The relative concentration of individual FA was expressed as percent of total FA equal to or greater than six carbons for diets and greater than 14 carbons for plasma and RBC membrane samples. In most instances, FA percentages of total below 0.2% have been excluded from tabulation. The FA analyses of formulae were performed by Mead-Johnson Laboratories and confirmed in our laboratory using methodologies based on procedures of the Association of Official Analytical Chemists (27). Briefly, lipids were extracted using ethanol/diethyl ether/petroleum hydrocarbon. Methyl esters were prepared as above under N_2 and extracted twice with 0.5 mL methylene chloride essentially according to MacGee and Allen (28). Recovery of short-chain FA in formulas was monitored by addition of 9:0 and 19:0 FA as internal standards (500 ng each), and evaporation steps were avoided after formation of methyl esters.

Integrated data from each gas chromatogram were filed and stored on an IBM AT computer. A customized software package was developed to allow for semi-automated identification of FA methyl esters, and accurate peak identification was verified by an experienced technician. The coded data were sorted and compiled into Lotus files for further inspection and statistical analysis.

Determination of thiobarbituric acid reactive substances (TBARS). Lipid peroxidation products (TBARS) from RBC exposed to H_2O_2 were quantitated by the method described by Cynamon *et al.* (29). TBARS were measured with and without sodium azide in the incubation mixture. Azide inhibits antioxidant enzymes (*e.g.*, catalase); thus a maximal release of TBARS is obtained. The percentage of the native TBARS concentration in RBC (− azide) divided by the maximal TBARS released (+ azide) gives a value that is normalized for individual biological variation. A high percentage value suggests a high capacity for intrinsic lipid peroxidation in the RBC membrane as has been demonstrated in vitamin E deficiency (29). Briefly, washed RBC are incubated with 0.75% H_2O_2 (+ azide, maximal TBARS) or 3% H_2O_2(− azide) for 1 h at 37°C. The reaction is terminated with 0.1 M arsenite in 2.8% trichloroacetic acid, color developed with 1% thiobarbituric acid/50 mM NaOH, and absorbancy measured at 535 nm. TBARS were quantitated by comparison to a tetraethoxypropane standard curve. Limited amounts of sample obtainable from the infants restricted the number of TBARS determinations. The "n" for the HM, Formula A, B and C groups was 7, 9, 9 and 8, respectively.

RBC fragility. The susceptibility of RBC from HM- and formula-fed infants to H_2O_2-induced hemolysis was determined according to the method described by Gordon *et al.* (30). Washed RBC were incubated at 37°C for 3 h with saline, water, and 2.4%, 8% or 10% H_2O_2. After centrifugation, released hemoglobin was measured spectrophotometrically at 540 nm. H_2O_2 values minus saline control values were divided by maximal hemoglobin release values (after H_2O_2 incubation) to give a percent of total hemolysis induced at different peroxide concentrations. The number of subjects studied was limited by the available amounts of blood.

Data analysis. Descriptive statistics were computed for all dependent variables in the analysis. Analysis of variance (ANOVA) was used to analyze the effects of the four diet groups at each of three times. Significant main effects were analyzed *post-hoc* using Newman-Keuls multiple comparison procedure with an α at 0.05. The sample size of 15 for each of the formula groups allowed us to detect a difference of 0.7 standard deviation (SD) between mean FA relative content with an α of 0.05 and a β of 0.8.

RESULTS

Characteristics of study groups. Twelve HM-fed infants and 83 formula-fed infants were entered into the study. No significant differences in birth weight, length, head circumference, gestational age or sex distribution were noted. Table 2 summarizes the main characteristics of the infants on entry and their growth throughout the study. The HM-fed infants were, however, slower in reaching 1800 g relative to the three formula-fed groups (ANOVA, $P < 0.07$). Growth, based on body weight, length and head circumference was essentially identical for the 4 groups upon completion of a 5 to 6 mon feeding regimen (57 wk postconception). Small losses (n = 3) in cohort population between entry and 36 wk (discharge from nursery) were due to illnesses which interfered with feeding regimens. One subject developed necrotizing enterocolitis, one had patent *ductus arteriosus* requiring surgery and another had feeding intolerance with feeding suspended for more than 24 h. However, there was a significant attrition of subjects during the 4–5 mon out-patient follow-up despite monthly appointments and direct involvement of the research nurse in promoting their return through repeated

TABLE 2

Characteristics of Study Subjects

Variable	Human milk	Formula A	Formula B	Formula C	ANOVA F	ANOVA P
Birth weight (g)[a]	1297 ± 131[b]	1327 ± 116	1273 ± 114	1299 ± 111	1.0	n.s.
Birth length (cm)[a]	39.0 ± 2.1	39.6 ± 1.6	39.0 ± 1.6	39.1 ± 1.8	0.6	n.s.
Birth head circumference (cm)[a]	27.2 ± 1.1	27.7 ± 1.2	27.2 ± 1.2	27.3 ± 1.3	0.8	n.s.
Gestational age (wk)[a]	30.1 ± 1.2	30.2 ± 1.7	29.9 ± 1.4	30.3 ± 1.5	0.5	n.s.
Days to 1,800 g[a]	35.1 ± 5.5[c]	28.8 ± 6.4	31.4 ± 7.8	30.5 ± 6.4	2.5	<.07
Weight at 57 wk (g)[d]	6114 ± 779	5902 ± 513	6045 ± 732	6357 ± 743	1.2	n.s.
Length at 57 wk (cm)[d]	61.0 ± 1.9	60.7 ± 2.0	60.4 ± 2.3	61.0 ± 2.2	0.2	n.s.
Head circumference at 57 wk (cm)[d]	41.0 ± 0.9	41.3 ± 1.3	41.1 ± 1.3	41.4 ± 1.4	0.3	n.s.

[a]The number of measurements (n) for each group was: Human milk, n = 12; Formula A, n = 25; B, n = 28; C, n = 30; F, follow-up; P, preterm; ANOVA, analysis of variance.

[b]Mean ± SD.

[c]Significantly different from formula groups using *post-hoc* Newman Keuls, $P < 0.05$.

[d]The number of measurements (n) for each group was: Human milk, n = 9; Formula A, n = 16; B, n = 18; C, n = 16.

phone calls. More than one-third of the patients did not complete the out-patient follow-up phase of the study.

Fatty acid analysis. Upon entry (10 d postnatally), the FA profiles for the total plasma and RBC lipids of the four cohorts were similar as shown in Tables 3 and 4. A notable exception was the elevated monounsaturated FA, primarily 18:1ω9, present in the plasma lipids of the HM-fed infants due to the higher concentration of oleic acid in HM received by these infants before the onset of the study. Similarly, the higher concentration of linoleic acid (18:2ω6) received by formula-fed infants prior to the study was reflected in their plasma lipids compared to that of the HM-group. In contrast, only slight differences were found for the 18:1 and 18:2 contents in the RBC lipids of the HM-group compared to formula-fed infants. A significant elevation in the content of a typical marker for EFA deficiency, 20:3ω9, was found in RBC lipids of the three formula-fed groups compared to HM-fed infants. Clinical EFA deficiency was not a consideration in these infants as the triene/tetraene ratio (20:3ω9/20:4ω6) was well below threshold values (31,32). Even upon entry to the study, selective effects of the ω3 FA in the HM-fed group compared to the formula-fed groups were evident as a slight elevation of DHA in the plasma lipid fraction. Other FA of the ω3 series were slightly reduced in the HM-group (Table 3) including an unexplained decrease in 20:5ω3 content, although all values were within published ranges (14,16,17). No differences between the four cohorts were observed in the FA profiles (except 20:3ω9) of total RBC lipids at entry (Table 4).

At an average of 36 wk post-conception, after 4 to 6 wk on study diets, major differences were found in the ω3 FA of both plasma and RBC lipids (Tables 5 and 6). Monounsaturated FA were elevated and 18:2ω6 was reduced in the plasma lipids of the HM-fed group compared to the formula groups, similar to that found at entry. The EFA deficiency marker, 20:3ω9, also remained significantly elevated in the RBC lipids of the three formula groups. The ω6 FA, 20:2 and 22:5, were significantly elevated in the plasma lipids of low ω3 FA group receiving Formula A. Slight enrichment of the other ω6 FA was evident in both plasma and RBC lipids of infants fed Formulas A and B. The order of mean values in ω3 FA concentrations in plasma and RBC lipids was generally: Formula C = HM > Formula B > Formula A. An important enrichment was observed in the DHA content of the plasma and RBC lipids of the infants receiving the diets containing ω3 LCP, HM and Formula C. Of additional interest were alterations in the end product/precursor ratios for both ω6 and ω3 FA desaturation pathways at this time point. Since the 18:2ω6, 18:3ω3 and 22:6ω3 contents vary in the four diets, a true comparison of metabolic conversion is confounded; however, similar 20:4ω6/18:2ω6 ratios for all diets in both plasma and RBC lipids suggests that metabolism of the ω6 series was unaffected by diet FA composition. In contrast, the ω3 FA conversion ratio (22:6ω3/18:3ω3) was significantly reduced in plasma and RBC lipids of infants fed Formula B compared to HM and Formulas A and C. However, interpretation of this ratio is complicated by the high proportion of 18:3ω3 in this formula.

Results of FA analysis from infants at 57 wk postconceptional age, receiving diets for 5–6 mon, presented very little difference in the monounsaturated FA of either plasma or RBC lipids (Tables 7 and 8). Despite the fact that all formulas contained a greater percentage of saturated FA (62–67%) than in HM (45%), both plasma and RBC lipids from HM-fed infants were enriched in saturates compared to formula groups, particularly the corn oil-based Formula A group. Of additional interest, the plasma and RBC lipid FA distributions of the HM- and Formula C-fed groups were remarkably similar, yet the ω6 EFA (18:2ω6) compositions of these two diets varied markedly, 12.7% *vs.* 20.4%.

In Formula A and B groups, the 18:2ω6, 22:5ω6 and 22:4ω6 content in plasma and RBC fractions were comparable but differed from that of the two groups receiving ω3 LCP (Formula C and HM). Studies of ω3 FA dietary enrichment in rats specifically implicate an inhibition of Δ6 desaturase as leading to a reduction of ω6 LCP in serum lipids (33). These observations are confirmed in long-term human infant studies (34). The ω3 FA composition of plasma and RBC lipids were lowest in the Formula A-fed group, intermediate in Formula B infants and essentially equivalent for the HM and marine oil (Formula C) groups as found at 36 wk. Similarly, a reduction in the ω3 product/precursor ratio (22:6ω3/18:3ω3) was found for

TABLE 3

Fatty Acid Composition of Total Plasma Lipids at Entry[a]

	Human milk (n = 12)	Formula A (n = 22)	Formula B (n = 26)	Formula C (n = 29)	ANOVA F	ANOVA P
Saturated	32.5 ± 3.1	32.4 ± 3.5	34.9 ± 3.5	34.5 ± 2.8	2.3	n.s.
Monounsaturated	26.7 ± 5.8[b]	19.9 ± 2.6	19.9 ± 2.4	19.9 ± 2.8	15.8	<0.001
20:3ω9	0.23 ± 0.12	0.28 ± 0.16	0.31 ± 0.15	0.34 ± 0.22	1.35	n.s.
ω6 FA						
18:2	25.3 ± 6.6[b]	31.2 ± 3.3	29.6 ± 4.2	29.5 ± 3.9	4.4	<0.01
20:2	0.73 ± 0.11	0.82 ± 0.21	0.80 ± 0.23	0.84 ± 0.26	0.7	n.s.
20:3	2.13 ± 0.56	2.00 ± 0.52	1.83 ± 0.55	2.03 ± 0.66	0.9	n.s.
20:4	8.0 ± 1.9	8.0 ± 1.6	7.4 ± 1.5	7.5 ± 1.6	0.7	n.s.
22:4	0.38 ± 0.09	0.39 ± 0.13	0.50 ± 0.21	0.54 ± 0.28	1.0	n.s.
22:5	0.38 ± 0.17	0.46 ± 0.25	0.47 ± 0.25	0.50 ± 0.19	0.8	n.s.
Σ ω6 LCP	11.64 ± 2.3	11.66 ± 2.17	10.97 ± 2.13	11.39 ± 2.2	0.4	n.s.
ω3 FA						
18:3	0.82 ± 0.78	1.33 ± 0.37	1.23 ± 0.62	1.20 ± 0.57	2.1	n.s.
20:5	0.33 ± 0.16[b]	0.56 ± 0.23	0.56 ± 0.16	0.60 ± 0.22	5.7	<0.001
22:5	0.29 ± 0.10	0.41 ± 0.15	0.40 ± 0.13	0.38 ± 0.14	2.1	n.s.
22:6	1.54 ± 0.50	1.48 ± 0.40	1.42 ± 0.38	1.41 ± 0.39	0.4	n.s.
Σ ω3 LCP	2.47 ± 0.57	2.55 ± 0.56	2.62 ± 0.63	2.56 ± 0.58	0.2	n.s.
20:4ω6/18:2ω6	0.35 ± 0.15[b]	0.26 ± 0.06	0.26 ± 0.08	0.26 ± 0.07	3.8	<0.05
22:6ω3/18:3ω3	3.86 ± 2.78[b]	1.19 ± 0.42	1.20 ± 0.47	1.33 ± 0.54	20.2	<0.001
Unsaturation index[b]	138 ± 9	146 ± 12	140 ± 10	142 ± 10	1.2	n.s.

[a]Mean ± SD; g/100 g lipids. All samples were taken at 10 d of age before formula groups started study diets; values with superscript b are significantly different by *post-hoc* Newman-Keuls, $P < 0.05$; F, follow-up; P, preterm; ANOVA, analysis of variance; LCP, long-chain polyunsaturated fatty acids.

[b]Sum of [number of double bonds for each fatty acid (FA) × % of each FA].

TABLE 4

Fatty Acid Composition of Total Red Blood Cell (RBC) Lipids at Entry[a]

	Human milk (n = 12)	Formula A (n = 20)	Formula B (n = 21)	Formula C (n = 28)	ANOVA F	ANOVA P
Saturated	42.8 ± 5.6	45.8 ± 6.8	42.7 ± 5.2	42.7 ± 6.0	1,5	n.s.
Monounsaturated	18.7 ± 3.5	17.4 ± 2.5	16.8 ± 2.4	18.0 ± 4.5	0.9	n.s.
20:3ω9	0.57 ± 0.17[b]	0.80 ± 0.32[b,c]	0.83 ± 0.26[b,c]	1.0 ± 0.62[c]	3.6	<0.05
ω6 FA						
18:2	8.8 ± 2.0	9.6 ± 1.8	9.7 ± 1.4	9.1 ± 1.7	1.0	n.s.
20:2	0.51 ± 0.13	0.60 ± 0.36	0.53 ± 0.19	0.55 ± 0.35	0.3	n.s.
20:3	2.54 ± 0.31	2.18 ± 0.46	2.09 ± 0.69	2.24 ± 1.10	0.6	n.s.
20:4	15.7 ± 4.2	13.8 ± 4.2	15.6 ± 5.0	15.1 ± 5.1	0.6	n.s.
22:4	3.06 ± 0.98	3.36 ± 1.27	4.04 ± 1.47	3.93 ± 1.68	1.6	n.s.
22:5	1.16 ± 0.62	1.41 ± 0.46	1.81 ± 1.07	1.78 ± 0.85	2.2	n.s.
Σ ω6 LCP	22.95 ± 5.47	21.36 ± 5.69	24.08 ± 6.48	23.5 ± 6.82	0.8	n.s.
ω3 FA						
18:3	0.10 ± 0.16	0.13 ± 0.10	0.25 ± 0.30	0.11 ± 0.09	2.9	n.s.
20:3	0.68 ± 0.38	0.44 ± 0.23	0.31 ± 0.35	0.52 ± 0.70	1.5	n.s.
20:5	0.29 ± 0.29	0.27 ± 0.18	0.34 ± 0.32	0.26 ± 0.20	0.3	n.s.
22:5	0.52 ± 0.43	0.48 ± 0.22	0.48 ± 0.22	0.53 ± 0.37	0.1	n.s.
22.6	4.06 ± 1.14	3.34 ± 1.44	4.19 ± 1.94	4.04 ± 1.89	1.0	n.s.
Σ ω3 LCP	5.86 ± 1.64	4.76 ± 1.29	5.48 ± 1.87	5.51 ± 1.99	1.1	n.s.
20:4ω6/18:2ω6	1.77 ± 0.45	1.49 ± 0.56	1.68 ± 0.65	1.72 ± 0.68	0.7	n.s.
22:6ω3/18:3ω3	25.8 ± 18.0	23.5 ± 13.6	26.7 ± 18.0	32.2 ± 19.5	0.9	n.s.
Unsaturation index[b]	160 ± 30	149 ± 28	166 ± 32	163 ± 34	1.2	n.s.

[a]Mean ± SD; g/100 g lipids. Details as in Table 3. Values with different superscripts (b,c) are significantly different by *post-hoc* Neuman-Keuls, $P < 0.05$.

[b]Sum of [number of double bonds for each fatty acid (FA) × % of each FA].

TABLE 5

Fatty Acid Composition of Total Plasma Lipids at 36 Wk Postconception[a]

	Human milk (n = 12)	Formula A (n = 21)	Formula B (n = 25)	Formula C (n = 27)	ANOVA F	ANOVA P
Saturated	34.6 ± 2.9	34.6 ± 4.4	33.4 ± 1.9	33.6 ± 2.7	0.8	n.s.
Monounsaturated	22.2 ± 5.7[b]	18.7 ± 2.5	17.8 ± 2.0	17.7 ± 2.1	7.4	<0.001
20:3ω9	0.12 ± 0.05	0.17 ± 0.08	0.16 ± 0.07	0.12 ± 0.08	2.0	n.s.
ω6 FA						
18:2	30.4 ± 5.0[b]	34.4 ± 4.4	34.6 ± 3.1	33.5 ± 2.1	4.4	<0.01
18:3	0.23 ± 0.08	0.29 ± 0.12	0.34 ± 0.13	0.28 ± 0.10	2.7	n.s.
20:2	0.72 ± 0.15[b]	1.00 ± 0.12[c]	0.82 ± 0.10[d]	0.76 ± 0.21[b]	21.4	<0.001
20:3	1.93 ± 0.65	1.79 ± 0.55	1.85 ± 0.43	1.88 ± 0.56	0.2	n.s.
20:4	5.39 ± 1.18	6.54 ± 1.95	6.21 ± 1.35	5.63 ± 1.58	2.1	n.s.
22:4	0.36 ± 0.17	0.56 ± 0.49	0.36 ± 0.18	0.48 ± 0.44	1.4	n.s.
22:5	0.24 ± 0.07	0.56 ± 0.19[b]	0.38 ± 0.20	0.28 ± 0.18	12.9	<0.001
Σ ω6 LCP	8.63 ± 1.66	10.45 ± 2.75	9.62 ± 1.70	9.04 ± 2.30	2.6	n.s.
ω3 FA						
18:3	0.67 ± 0.24[b]	0.25 ± 0.09[c]	1.73 ± 0.40[d]	0.80 ± 0.17[b]	135.2	<0.001
20:5	0.81 ± 0.65[b]	0.10 ± 0.06[c]	0.38 ± 0.13[d]	1.39 ± 0.52[e]	54.1	<0.001
22:5	0.44 ± 0.20[b]	0.21 ± 0.09[b]	0.47 ± 0.16[d]	0.68 ± 0.25[d]	28.8	<0.001
22:6	1.68 ± 0.86[b]	0.59 ± 0.30[c]	1.28 ± 0.25[d]	2.61 ± 0.99[e]	61.4	<0.001
Σ ω3 LCP	3.15 ± 1.58[b]	1.14 ± 0.39[c]	2.30 ± 0.43[d]	4.88 ± 1.59[e]	67.6	<0.001
20:4ω6/18:2ω6	0.18 ± 0.06	0.19 ± 0.05	0.18 ± 0.05	0.17 ± 0.06	1.6	n.s.
22:6ω3/18:3ω3	2.67 ± 1.23	2.66 ± 2.11	0.82 ± 0.40[b]	3.52 ± 1.80	14.4	<0.001
Unsaturation index[b]	135 ± 13[b,c]	134 ± 15[c]	142 ± 7[b,d]	148 ± 13[d]	5.9	<0.001

[a]Mean ± SD; g/100 g lipid. All samples taken between 35 and 37 wk postconception. Details as in Table 3. Values with different superscripts (b,c,d,e) are significantly different by *post-hoc* Newman-Keuls, $P < 0.05$.

[b]Sum of [number of double bonds for each fatty acid (FA) × % of each FA].

TABLE 6

Fatty Acid Composition of Total Red Blood Cells Lipids at 36 Wk Postconception[a]

	Human milk (n = 12)	Formula A (n = 20)	Formula B (n = 21)	Formula C (n = 27)	ANOVA F	ANOVA P
Saturated	39.7 ± 8.0	43.0 ± 6.2	41.0 ± 4.7	40.3 ± 3.9	0.6	n.s.
Monounsaturated	19.2 ± 5.6[b]	15.4 ± 1.9	14.9 ± 2.2	15.5 ± 1.7	4.4	<0.01
20:3ω9	0.17 ± 0.19[b]	0.57 ± 0.42	0.47 ± 0.25	0.42 ± 0.29	3.4	<0.05
ω6 FA						
18:2	11.1 ± 1.6[b]	12.4 ± 1.6	12.8 ± 1.5	12.5 ± 1.3	3.6	<0.05
20:2	0.76 ± 0.22[b,c]	0.89 ± 0.19[b]	0.78 ± 0.19[b,c]	0.66 ± 0.16[c]	5.9	<0.001
20:3	2.77 ± 0.58	2.59 ± 0.61	2.44 ± 0.52	2.41 ± 0.61	1.2	n.s.
20:4	14.3 ± 5.3	14.4 ± 3.6	15.7 ± 3.1	14.8 ± 2.3	1.0	n.s.
22:4	3.27 ± 1.61	4.47 ± 1.65	4.18 ± 1.09	4.13 ± 1.69	0.9	n.s.
22:5	0.77 ± 0.62[b]	1.65 ± 0.56[c]	1.45 ± 0.45[c,d]	1.27 ± 0.48[d]	7.8	<0.001
Σ ω6 LCP	21.85 ± 7.04	24.00 ± 5.72	24.58 ± 4.57	23.23 ± 3.30	0.7	n.s.
ω3 FA						
18:3	0.09 ± 0.15	0.08 ± 0.10	0.22 ± 0.17[b]	0.09 ± 0.07	5.8	<0.001
20:3	0.89 ± 1.21	0.45 ± 0.28	0.39 ± 0.41	0.36 ± 0.50	2.3	n.s.
20:5	0.80 ± 0.97[b]	0.15 ± 0.18[c]	0.36 ± 0.25[c]	0.91 ± 0.41[b]	14.0	<0.001
22:5	1.83 ± 0.91	0.73 ± 0.38[b]	1.35 ± 0.56	1.69 ± 0.52	14.3	<0.001
22:6	3.94 ± 2.55[b,c]	2.71 ± 1.13[d]	3.56 ± 1.17[c]	4.71 ± 1.31[b]	8.7	<0.001
Σ ω3 LCP	7.64 ± 3.48[b]	4.39 ± 1.23[c]	5.85 ± 1.39[d]	7.93 ± 1.71[b]	16.6	<0.001
20:4ω6/18:2ω6	1.30 ± 0.47	1.16 ± 0.24	1.24 ± 0.26	1.20 ± 0.26	1.3	n.s.
22:6ω3/18:3ω3	55.5 ± 50.8	37.9 ± 19.3	15.4 ± 7.2[b]	37.8 ± 10.0	8.9	<0.001
Unsaturation index[b]	167 ± 40	159 ± 28	170 ± 24	176 ± 19	1.9	n.s.

[a]Mean ± SD, g/100 g lipid. All samples taken between 35 and 37 wk postconception. Details as in Table 3. Values with different superscripts (b,c,d) are significantly different by *post-hoc* Newman-Keuls, $P < 0.05$.

[b]Sum of [number of double bonds for each fatty acid (FA) × % of each FA].

TABLE 7

Fatty Acid Composition of Total Plasma Lipids at 57 Wk Postconception[a]

	Human milk (n = 8)	Formula A (n = 13)	Formula B (n = 16)	Formula C (n = 14)	ANOVA F	ANOVA P
Saturated	34.0 ± 2.4	30.6 ± 2.4[b]	32.7 ± 2.3	33.4 ± 1.9	4.9	<0.005
Monounsaturated	16.8 ± 3.6	16.7 ± 1.4	14.8 ± 2.0	15.3 ± 2.1	2.5	n.s.
20:3ω9	0.11 ± 0.16	0.10 ± 0.05	0.06 ± 0.06	0.09 ± 0.18	0.4	n.s.
ω6 FA						
18:2	36.2 ± 6.1[b]	43.3 ± 2.4[c]	40.7 ± 2.2[c]	37.7 ± 4.17[b]	8.2	<0.001
18:3	0.20 ± 0.11	0.16 ± 0.04	0.15 ± 0.05	0.16 ± 0.08	1.0	n.s.
20:2	0.72 ± 0.64	0.64 ± 0.06	0.59 ± 0.09	0.48 ± 0.10	1.6	n.s.
20:3	1.38 ± 0.53	1.11 ± 0.27	1.19 ± 0.37	1.06 ± 0.34	1.4	n.s.
20:4	4.57 ± 1.46	4.88 ± 1.23	5.26 ± 1.29	4.03 ± 1.55	1.9	n.s.
22:4	0.30 ± 0.15	0.67 ± 0.53	0.54 ± 0.27	0.33 ± 0.26	3.0	n.s.
22:5	0.27 ± 0.22[b,c]	0.41 ± 0.13[b]	0.27 ± 0.14[b,c]	0.16 ± 0.18[c]	5.1	<0.005
Σ ω6 LCP	7.57 ± 2.43	7.71 ± 1.68	7.84 ± 1.75	6.10 ± 2.20	2.2	n.s.
ω3 FA						
18:3	0.87 ± 0.19[b,c]	0.62 ± 0.73[c]	1.67 ± 0.53[d]	1.17 ± 0.34[b]	10.6	<0.001
20:5	1.12 ± 0.51[b]	0.06 ± 0.06[d]	0.24 ± 0.09[d]	1.64 ± 0.39[c]	84.8	<0.001
22:5	0.82 ± 0.18[b]	0.20 ± 0.08[c]	0.60 ± 0.23[d]	0.82 ± 0.22[b]	27.4	<0.001
22:6	2.47 ± 0.54[b]	0.41 ± 0.20[c]	1.07 ± 0.22[d]	3.22 ± 0.80[e]	79.0	<0.001
Σ ω3 LCP	4.72 ± 1.26[b]	0.84 ± 0.37[c]	2.11 ± 0.47[d]	5.87 ± 1.28[e]	86.5	<0.001
20:4ω6/18:2ω6	0.14 ± 0.09	0.11 ± 0.03	0.13 ± 0.04	0.12 ± 0.07	0.6	n.s.
22:6ω3/18:3ω3	2.94 ± 0.67[b]	1.02 ± 0.54	0.75 ± 0.43	2.85 ± 0.87[b]	41.4	<0.001
Unsaturation index[b]	148 ± 11	139 ± 6	142 ± 7	150 ± 7[b]	6.5	<0.001

[a]Mean ± SD, g/100 g lipid. All samples taken at 57 wk postconception. Details as in Table 3. Values with different superscripts (b,c,d,e) are significantly different by *post-hoc* Newman-Keuls, $P < 0.05$.

[b]Sum of [number of double bonds for each fatty acid (FA) × % of each FA].

TABLE 8

Fatty Acid Composition of Total Red Blood Cell Lipids at 57 Wk Postconception[a]

	Human milk (n = 8)	Formula A (n = 13)	Formula B (n = 16)	Formula C (n = 13)	ANOVA F	ANOVA P
Saturated	45.6 ± 3.6	40.7 ± 3.2	42.6 ± 6.8	43.7 ± 7.0	1.4	n.s.
Monounsaturated	15.2 ± 1.9[b]	13.2 ± 1.2[c]	12.9 ± 2.0[c]	13.9 ± 1.6[b,c]	4.5	<0.01
20:3ω9	0.12 ± 0.16	0.11 ± 0.08	0.07 ± 0.07	0.31 ± 0.48	2.6	n.s.
ω6 FA						
18:2	14.3 ± 3.1[b]	17.8 ± 1.4[c]	17.0 ± 1.3[c]	15.2 ± 2.3[b]	7.3	<0.001
20:2	0.61 ± 0.15	1.02 ± 0.21	0.89 ± 0.42	1.55 ± 2.01	1.8	n.s.
20:3	1.95 ± 0.54	2.14 ± 0.60	1.78 ± 0.67	1.74 ± 0.55	1.4	n.s.
20:4	10.8 ± 2.3[b,c]	14.0 ± 1.9[c]	13.7 ± 3.9[c]	9.3 ± 3.1[b]	7.5	<0.001
22:4	2.17 ± 0.48[b]	6.0 ± 1.5[c]	4.67 ± 1.74[d]	2.04 ± 0.59[b]	26.3	<0.001
22:5	0.39 ± 0.14	1.74 ± 0.7[b]	0.85 ± 0.29	0.42 ± 0.26	24.0	<0.001
Σ ω6 LCP	15.93 ± 2.88[b]	24.91 ± 3.36[c]	21.90 ± 6.01[c]	15.06 ± 4.26[b]	13.1	<0.001
ω3 FA						
18:3	0.12 ± 0.05	0.17 ± 0.27	0.23 ± 0.11	0.26 ± 0.48	0.5	n.s.
20:3	0.36 ± 0.29	0.38 ± 0.38	0.31 ± 0.55	0.25 ± 0.29	0.2	n.s.
20:5	1.01 ± 0.68[b]	0.24 ± 0.11[c]	0.46 ± 0.22[c]	1.82 ± 0.78[d]	24.7	<0.001
22:5	2.51 ± 0.82	1.07 ± 0.69[b]	2.16 ± 0.59	2.86 ± 1.17	10.0	<0.001
22:6	4.46 ± 1.66[b]	1.15 ± 0.77[c]	2.41 ± 0.86[c]	5.88 ± 3.08[b]	15.8	<0.001
Σ ω3 LCP	8.61 ± 2.75[b]	3.05 ± 1.42[c]	5.33 ± 1.47[d]	11.30 ± 4.20[e]	21.6	<0.001
20:4ω6/18:2ω6	0.83 ± 0.39	0.79 ± 0.12	0.79 ± 0.21	0.61 ± 0.19	2.3	n.s.
22:6ω3/18:3ω3	34.0 ± 11.0[b]	13.2 ± 6.1[c]	8.56 ± 2.76[c]	41.4 ± 39.1[b]	6.8	<0.001
Unsaturation index[b]	152 ± 20	162 ± 15	161 ± 31	163 ± 37	1.0	n.s.

[a]Mean ± SD, g/100 g lipid. All samples taken at 57 weeks postconception. Details as in Table 3. Values with different superscripts (b,c,d,e) are significantly different by *post-hoc* Newman-Keuls, $P < 0.05$.

[b]Sum of [number of double bonds for each fatty acid (FA) × % of each FA].

Formula B infants. Group differences in 20:3ω9 were no longer significantly different by 57 wk postconception.

The ratio of end-products of the ω3 (DHA) and ω6 (DPA) FA desaturation (namely, 22:6ω3/22:5ω6, DHA to DPA) have been compared for each dietary regimen as a measure of the dietary ω3 FA sufficiency (Fig. 1). This ratio of sufficiency after 36 wk was significantly elevated in RBC total lipids of infants receiving the HM and marine oil diets (Formula C) relative to infants receiving the two formulas lacking in ω3 LCP (A and B). At 57 wk, the sufficiency ratios in HM- and Formula C-fed infants were nearly identical and both were significantly elevated above that of the corn (A) and soy (B) oil formulas. At 36 wk, the infants receiving α-linolenic acid-rich Formula (B) were able to maintain their 22:6ω3 at a level equivalent to the HM and marine oil (C) groups (Tables 5 and 6); however, the balance of ω3 to ω6 LCP was reduced. By 57 wk, the infants receiving 2.7% α-linolenic acid (Formula B) were unable to synthesize sufficient 22:6ω3 and a compensatory elevation in 22:5ω6 was evident from the ratio. The 22:6ω3/22:5ω6 ratio in total plasma lipids paralleled that of the RBC lipids at 36 and 57 wk.

Lipid peroxidation and RBC fragility. The lipid peroxide products, TBARS, were measured in the RBC of the four study groups (Table 9). The native TBARS concentration was measured in RBC after 3% H_2O_2 treatment. Maximal release of TBARS from RBC was determined after treatment with 0.75% H_2O_2 and azide, to block antioxidant enzymes. Native TBARS concentrations (− azide) in the HM- and formula-fed groups were essentially identical whereas the total TBARS concentrations (+ azide) generated by the LCP-enriched groups (HM and Formula C) were not significantly greater than in infants receiving formulas A and B lacking ω3 LCP. The trend to higher values may reflect the greater unsaturation in these formula groups. The lower percentage of maximal TBARS release in the LCP-enriched groups may reflect a lower potential for lipid peroxidation in the RBC membranes due to higher antioxidant contents of these diets. In a *post-hoc* analysis combining the two ω3 LCP supplemented groups (Formula C and HM) as compared to the combined Formulas A and B groups, the difference becomes significant, $P < 0.05$.

No significant (ANOVA, $P < 0.05$) differences in the fragility of the RBC membranes was found between diet groups when RBC were treated with 2.5% or 8–10% H_2O_2. The mean ± SE percent hemolysis observed with 2.5% H_2O_2 for groups receiving Formula A (n = 6), B (n = 8) and C (n = 6) were 5.5 ± 0.9, 11.3 ± 2.2, and 8.1 ± 2.5%, respectively. The percentages of hemolysis when RBC were treated with 8 to 10% H_2O_2 were 9.1 ± 2.2, 11.5 ± 4.2 and 14.9 ± 2.2%, respectively for the three formula groups.

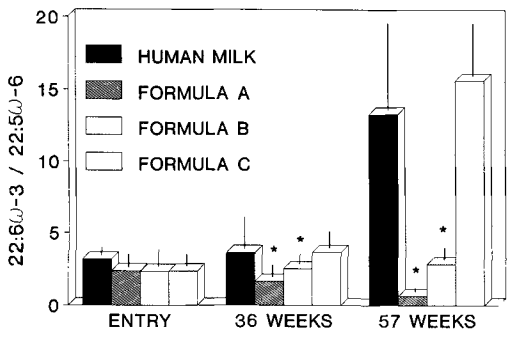

FIG. 1. Ratio of end products of ω3 and ω6 fatty acid (FA) metabolism (22:6ω3/22:5ω6) in red blood cells (RBC) total lipids. The 22:6ω3/22:5ω6 ratios are given for entry of infants into study (10 d postnatal age), 36 wk postconceptional age (~5 wk on study diets), and 57 wk postconceptional age (~6 mon on diets). See Materials and Methods for formula description. *Values are significantly different from other values by analysis of variance, $P < 0.001$. Bars and posts are means ± SD.

DISCUSSION

This study confirms previous observations that dietary EFA composition affects the relative content of ω3 and ω6 LCP in plasma and RBC lipids in infants (16,35,36) and supports an essential role for ω3 FA for premature infants. The corn-oil based formula (Formula A) is associated with important compositional changes of plasma and RBC lipids within a few weeks of study. The decreased content of DHA and elevation of 22:5ω6 suggest that ω3 deficiency exists in this group. The effects become more pronounced at 57 wk. The soy oil-based formula (Formula B) provides sufficient α-linolenic acid; however, infants fed this formula had a lower DHA content in plasma and RBC at 57 wk than those given HM or Formula C. These results suggest that despite the enhanced provision of substrate for Δ6 desaturation and a low ω6 FA/ω3 FA ratio in formula, provision of pre-formed ω3 LCP in the infants' formula diet is necessary to achieve a lipid profile comparable to that in HM. The supplementation of the soy oil formula with marine oils provides the ω3 LCP to obtain a FA profile in plasma and RBC lipids

TABLE 9

Thiobarbituric Acid Reactive Substances in Red Blood Cells (RBC) of Infants on Study Diets at 57 Wk Postconception[a]

	TBARS (nmol/mL packed RBC, mean ± SE)				ANOVA	
	HM	Formula A	Formula B	Formula C	F	P
− Azide	37.5 ± 9.8	36.2 ± 5.4	30.4 ± 3.9	32.8 ± 5.7	0.3	n.s.
+ Azide	629 ± 105	551 ± 121	442 ± 52	644 ± 80	1.0	n.s.
$\frac{-\text{ Azide}}{+\text{ Azide}} \times 100$ (%)	5.93 ± 1.42	7.42 ± 1.03	7.24 ± 0.97	5.33 ± 1.00	0.9	n.s.

[a]HM, human milk. TBARS, thiobarbituric acid reactive substance. Abbreviations as in Table 3.

close to that in the human milk-fed group. Although the marine oil-enriched formula contains an EPA content in excess of DHA, this relationship is not reflected *in vivo* in plasma or RBC lipids. This excess of ω3 FA in Formula C appears to decrease the arachidonic acid content of RBC membranes after several months of feeding but not after 4–5 wk.

The functional implications of these findings are not the subject of this report, but it is relevant to indicate that the FA compositions of plasma and RBC in these infants are significantly correlated with measures of retinal function and visual acuity maturation as we have previously reported (18,37,38). The small nonsignificant effects on indices of lipid peroxidation and RBC fragility indicate that this level of marine oil supplementation is not associated with major adverse effects on RBC membranes.

The purpose of this study was to evaluate the potential alternatives for supplementing preterm infant formulas with sources of ω3 FA. The comparison of the soy and soy/marine oil blends was included since there are questions as to the capacity of low-birth-weight infants to elongate and desaturate α-linolenic acid. A recent study of low-birth-weight infants (14) suggests that α-linolenic acid is sufficient to achieve an adequate DHA content in plasma and RBC phospholipids. Our data confirm this finding at 36 wk postconception; however, by 57 wk, α-linolenic acid is insufficient to maintain DHA levels in plasma and RBC lipids comparable to those found in infants fed ω3 LCP. The use of marine oils as a source of ω3 LCP in infant formula has been debated for some time. Previous studies have evaluated the use of marine oil supplements (17) and egg phospholipids (16) as sources of ω3 LCP over several weeks of feeding. This current study evaluated the inclusion of marine oil ω3 LCP into formula for a prolonged period of time and documented the possibility of maintaining DHA levels postnatally. In addition, significant associations between low DHA levels and delays in both retinal electrophysiologic and visual acuity development (18,37,38) were found when infants are provided formula lacking ω3 LCP. The possibility of accumulation of EPA has been raised by some investigators yet our data confirm studies (39) in the ω3 deficient chick indicating that there is active conversion of EPA to DHA and the modest accumulation of EPA in plasma and tissue lipids that we observed is without significant adverse effects. Our results suggest that EPA is metabolized to DHA since the ratio of EPA to DHA in Formula C was 2 to 1 in comparison to that in the infant's RBC lipids of 1 to 3.2. This conclusion is further supported by ω3 and ω6 FA product/precursor ratios for the Δ4 desaturase (*i.e.*, 22:6ω3/22:5ω3 and 22:5ω6/22:4ω6). The ratios for desaturation of the ω3 and ω6 series of FA were similar for the HM- and marine oil-fed infants (1.78 and 0.18 for HM *vs.* 2.04 and 0.20 for Formula C, respectively) and agree with the results of Koletzko *et al.* (16).

Interpretation of product/precursor ratios as indices of desaturase activity is complicated by inclusion of either product or precursor FA in the study diets. However, careful evaluation of these indicators of desaturase activity would suggest that sufficient LCP can be produced by an active Δ4 desaturase, but production of its precursor by Δ6 desaturation may be the limiting factor. A similar conclusion was reached by Koletzko *et al.* (16). This restriction is readily avoided by a supply of LCP in the infants' diet.

The decrease in arachidonic acid in RBC of the marine oil group compared to the corn and soy oil groups observed at the 57 wk follow-up (Table 8) is of interest since Carlson *et al.* (34) have reported that low arachidonic acid levels may be associated with poor growth in formula-fed preterm infants. Although the RBC arachidonic acid content at 57 wk postconceptional age was reduced in the marine oil group, no differences among the three randomized groups in weight, length and head size were noted. The adverse effects on growth may not become evident unless prolonged supplementation is employed as in the study of Carlson and colleagues (34) where infants were fed marine-oil containing formula for 9 mon. The long-term safety of using marine oil in formulae and the optimal duration of ω3 LCP supplementation should be addressed by future studies. The potential need for arachidonic acid supplementation of formulas that have soy oil or marine oil sources added is supported by our data and is in accordance with the known competition of both series of EFA as substrates for the desaturases (1). If human milk is considered as the model for EFA supply in early life, both ω3 and ω6 LCP should be provided by the formula. Formulas used to feed premature infants should reflect the newly acquired knowledge on the EFA needs of this group of infants at known risk for improper development.

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Addition of Long-Chain Polyunsaturated Fatty Acids to Formula for Very Low Birth Weight Infants[1]

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Thirty-four premature infants who were appropriate for gestational age and weighing less than 1500 g at birth were fed "preemie" SMA-24 formula, "preemie" SMA-24 formula manufactured to contain C_{20} and C_{22} ω6 and ω3 fatty acids (LCPE-SMA), or expressed milk (EBM). Blood samples were drawn from a small arm vein during the first week of life and after 28 days of feeding. The fatty acid content of plasma phospholipids was determined. Infants fed SMA-24 had a high content of 18:2ω6 in plasma phospholipids. Feeding LCPE-SMA normalized plasma phospholipid levels of C_{20} and C_{22} ω6 and ω3 fatty acids to be similar to levels of C_{20} and C_{22} ω6 and ω3 fatty acids found in infants fed EBM, and significantly higher than characteristic levels for infants fed SMA-24. Feeding LCPE-SMA or EBM results in a balanced incorporation of C_{20} and C_{22} ω6 and ω3 fatty acids into phospholipids derived from the liver or perhaps the small intestine. ***Lipids 27*, 896-900 (1992).**

Metabolism of long-chain polyunsaturated fatty acids derived from 18:2ω6 and 18:3ω3 by chain elongation-desaturation is essential for synthesis of complex structural lipids, leukotrienes, thromboxanes and prostaglandins. These essential fatty acids are required for normal function in developing tissues and appropriate maturation of a wide variety of physiological processes. During development, fetal accretion of long-chain metabolites of ω6 and ω3 fatty acids may result from maternal or placental synthesis and transfer or, alternatively, from the metabolism of 18:2ω6 and 18:3ω3 to longer chain homologues by the fetus (1). After birth the infant must synthesize or be fed the very long-chain polyunsaturated fatty acids of C_{20} and C_{22} type derived from 18:2ω6 and 18:3ω3. Limitation of this metabolic capability would be most critical to very low birth weight infants due to very low fatty acid reserves.

Metabolism of ω6 and ω3 fatty acids utilizes the same enzyme system and is competitive. When levels of dietary ω3 and ω6 C_{18} fatty acids are altered, the levels of metabolites of these precursor fatty acids change in specific brain membranes, influencing membrane lipid dependent functions (2-5). A diet balanced in very long-chain ω3 and ω6 fatty acids may increase brain membrane ω3 fatty acid content when 20:5ω3 is fed, while decreasing membrane fatty acid content of the ω6 series of competing fatty acids. As 20:4ω6 is quantitatively and qualitatively important to brain phospholipid (6), significant reduction in brain levels of 20:4ω6 may be less than optimal.

TABLE 1

Long-Chain Polyunsaturated Fatty Acids Required for a Physiological Intake Similar in Composition to Human Milk and That Provided by Modified SMA

Fatty acids	Range	Breast milk[a]	Modified SMA
C_{20} and C_{22} ω6	0.13 to 5.6%	1.0 ± 0.2	0.81
C_{20} and C_{22} ω3	0.013 to 3.3%	0.7 ± 0.2	0.7
Ratio of C_{20} and C_{22} ω6 to ω3		1.4 ± 2.0	1.14
18:2ω6		12 ± 4.3	14.1
18:3ω3		0.9 ± 0.4	1.6

[a]Ref. 6.

Human milk is generally believed to provide an optimal balance of fatty acids, particularly of essential fatty acids (6-8). During fetal development, C_{20} and C_{22} ω6 and ω3 fatty acids are utilized in the synthesis of new tissue membranes (6,8,9). Research has emphasized the need to provide these essential membrane components by maintaining the proper balance of 18:2ω6 to 18:3ω3, and by addition of C_{20} and C_{22} fatty acids of either ω6 or ω3 type to infant formula (6,8-12). With one exception (12), previous studies have supplemented low birth weight infant formulas with only C_{20} and C_{22} ω3 fatty acids derived from fish oils (11,13,14). When low birth weight infants have been fed formulas supplemented with C_{20} and C_{22} ω3 fatty acids, these fatty acids are apparently incorporated into red blood cell membrane lipids (11,13) and are probably incorporated into brain and other tissues, but the degree to which these fatty acids alter neutral membranes when fed in the diet as compared to controls is not known (15).

Infant formulas presently marketed do not contain longer chain polyunsaturated homologues of 18:2ω6 or 18:3ω3. We have suggested that these longer chain homologues should only be incorporated into low birth weight infant formulas in a manner and composition reflecting the consistent overall fatty acid balance found in the fatty acid mixture present in human milk (8,9; Table 1). Supplementation of feeds with only very long-chain ω3 fatty acids, although easily achieved, may not be desirable because it would reduce synthesis, metabolism and incorporation of 20:4ω6 into membrane phospholipids. Testing of balanced fat mixtures containing both ω6 and ω3 very long-chain polyunsaturated fatty acids is thus cogent, given the lack of information on neonatal ability to chain elongate and desaturate essential fatty acids. The present paper reports the effect of feeding low birth weight infants a triglyceride mixture providing a balanced fatty acid profile with a C_{20} and C_{22} ω6 and ω3 fatty acid content characteristic of human milk.

MATERIALS AND METHODS

All procedures concerning use of human subjects in this study were approved by the Human Experimentation

[1]Based on a paper presented at the Symposium on Milk Lipids held at the 1990 AOCS Annual Meeting, Baltimore, MD, April 1990.

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Abbreviations: EBM, expressed breast milk; GLC, gas-liquid chromatography; LCPE-SMA, SMA-24 formula manufactured to contain long-chain polyenoic essential fatty acids.

TABLE 2

Subject Descriptive Data at Birth[a]

	Group 1 EBM[b] (12)[e]	Group 2 LCPE-SMA[c] (12)	Group 3 SMA-24[d] (10)
Gestational age	30.1 ± 2.6	30.9 ± 1.2	31.0 ± 2.6
Birth weight (g)	1193 ± 257	1383 ± 150	1312 ± 230
Males	7	6	5
Females	5	6	5

[a]Values illustrated represent the mean ± SD.
[b]EBM, expressed breast milk.
[c]LCPE-SMA, long-chain polyenoic essential fatty acids added to SMA-24 formula.
[d]SMA-24, "preemie" infant formula.
[e]Number of infants in group.

Review Committee of the University of Alberta Hospitals (Canada).

Study population and feeding design. Thirty-four premature infants who were appropriate for gestational age and weighing less than 1500 g at birth were selected from admissions to the Neonatal Intensive Care Unit of the University of Alberta Hospitals. Informed written parental consent was obtained for all infants. Infants were divided, based on maternal preference, into human milk or formula groups. Infants were allocated into the formula groupings of with or without added ω6 and ω3 long-chain polyunsaturated fatty acids on a randomized basis. All infants were considered healthy upon enrollment. Infants included were those receiving oral nasogastric feedings within the first day of life and who were medically stable, without major respiratory or metabolic illness or severe congenital malformations. Gestational age was confirmed by Dubowitz score (16) and, in most cases, by early ultrasound dating.

Infants studied (Table 2) attained full feeds of 150 mL/kg/day by day 5 *postpartum*. Seven male and five female infants received their own mothers' milk up to 180–200 mL/kg/day (Group 1). Preterm expressed mothers' milk (EBM) was prepared in the hospital or at home, frozen, and delivered to the neonatal unit within 24 h. Five male and five female infants were fed "preemie" SMA-24 formula (SMA-24, Group 3), and six male and six female infants received "preemie" SMA-24 formula manufactured to contain C_{22} and C_{22} ω6 and ω3 fatty acids (LCPE-SMA, Group 2; Table 2). Infants receiving formula were fed apparent rates of energy intake approximately similar to those for infants fed human milk. All infants were strictly maintained on their particular feeding protocol. Introduction of any other source of fat intake resulted in loss of that infant from the study. Infants requiring transfusions were excluded from the study. Infants received vitamin (Tri-visol) and mineral supplements given routinely in the neonatal intensive care unit. Supportive therapy was provided as required in the form of aminophylline, phototherapy and, in some cases, oxygen. Infants were nursed predominantly on their sides and in an elevated position, in temperature controlled incubators at thermoneutral temperature with 60% humidity.

SMA-24 (Wyeth-Ayerst Laboratories, Radnor, PA) was chosen because it provides a fatty acid profile similar to that of human milk. A second formulation (Wyeth-Ayerst) of similar overall composition was prepared to contain an appropriate level of C_{20} and C_{33} ω6 and ω3 fatty acids (Table 3). This formulation was made by incorporating a fish oil triglyceride preparation containing C_{20} and $C_{22}2$ ω6 and ω3 fatty acids into the fat blend. The balance between C_{20} and C_{22} ω6 to C_{20} and C_{22} ω3 fatty acids was approximately 1.14 (see Tables 1 and 3).

TABLE 3

Fatty Acid Composition of SMA-24 and SMA Modified to Contain C_{20} and C_{22} ω6 and ω3 Fatty Acids

	Formula[a]	
Fatty acids (% w/w)	LCPE-SMA	SMA-24
6:0	0	0.2
8:0	5.1	8.3
10:0	3.3	4.3
12:0	9.7	13.2
14:0	5.1	5.5
16:0	13.2	11.5
16:1ω7	2.0	0.7
18:0	3.6	6.3
18:1ω9	35.0	35.4
18:2ω6	14.1	14.9
18:3ω3	1.6	1.6
20:0	0.2	0.2
20:1ω9	0.8	0.1
20:3ω6	0.3	—
20:4ω6	0.21	—
20:5ω3	0.2	—
22:0	0.1	0.2
22:1ω9	0.06	—
22:2ω6	0.04	—
22:4ω6	0.04	—
22:5ω6	0.07	—
22:5ω3	0.16	—
22:6ω3	0.35	—
24:0	0.04	—
24:1ω9	0.07	—

[a]As in footnotes [c] and [d] of Table 2.

Crown-heel length and occipitofrontal head circumference were measured on entry to the study and weekly. Body weight measurement was also made on entry, and daily weight was recorded for the first five weeks. All anthropometric measurements were made by one research nurse with an assistant.

A blood sample (1.0 mL) was obtained from each infant during the first week of life and after four weeks of feeding. Blood samples obtained from a small arm vein were immediately placed in ice, then centrifuged to separate plasma from blood cells. Both plasma and red blood cells were stored at −70°C under nitrogen until analysis.

Lipid analysis. Lipids were extracted from erythrocytes (17) and plasma (18), and antioxidant (1–2 μg of 2-ethoxyquin) was added to each sample. Internal standards (15:0, 17:0 and 19:0 as phosphatidylcholine, triglyceride and cholesteryl ester) were added to quantitate fatty acid content of each lipid class. Phospholipids were separated and detected under UV light with appropriate standards (19). Individual lipids were scraped from plates and quantitatively analyzed for fatty acid content.

Phospholipid fatty acid methyl esters were prepared using 14% (wt/vol) BF_3/methanol reagent (20). Fatty acid methyl esters were separated and quantitated by automated gas-liquid chromatography (GLC; Vista 6000 GLC and Vista 654 data system; Varian Instruments, Georgetown, Ontario, Canada). Chromatography was performed

using a fused silica BP20 capillary column (25 m × 0.25 mm i.d.; Varian; ref. 5). The analytical conditions used separate all *cis* saturated-, mono-, di- and polyunsaturated fatty acids from C_8 to C_{24} carbons long.

Statistical analysis. Statistical analyses included a preliminary one-way analysis of variance followed by multiple regression analysis, and analysis of variance to assess effect of feeding and postnatal age. Differences were determined using a multiple range comparison (21).

RESULTS AND DISCUSSION

In North America, marketed infant formulas do not contain the longer polyunsaturated homologues of 18:2ω6 and 18:3ω3. Balanced inclusion of these longer chain homologues (Table 1) in formula fat blends has proven problematic as sources of oil high in C_{20} and C_{22} ω3 fatty acids contain little or no 20:4ω6 or other C_{20} and C_{22} ω6 fatty acids. Thus, dietary studies have emphasized addition of fish oil, MAXEPA (11,14). Most marine oil sources can be ruled out because they are too high in ω3 fatty acids. Fresh water cat fish, however, is a source of high quality oil that contains a close to optimal balance of very long-chain polyenoic essential fatty acids after triglyceride purification. When combined with appropriate levels of other triglyceride components of SMA-24, an appropriate fatty acid balance was achieved (modified-SMA; Table 3). This modified SMA formula contained approximately one-third to one-half the 20:4ω6 level typical of human milk, significant levels of other C_{20} and C_{22} ω6 fatty acids, an approximately equal balance between 20:4ω6 and 20:5ω3, and an overall ratio of C_{20} and C_{22} ω6 fatty acids to C_{20} and C_{22} ω3 fatty acids of approximately 1.14 (Tables 1 and 3).

The present study was not designed to specifically measure growth in body constituents. No apparent differences were observed in birth weight, length, head circumference or gestational age between EBM and formula fed infants (Table 2). Phototherapy, sodium bicarbonate or antibiotic therapy, and oxygen requirements were similar for the three feeding groups. Clinical laboratory studies, such as acid base status, plasma and urine electrolytes, and total plasma proteins and calcium remained within normal limits. The initial sample of blood was drawn on approximately day 5 of life (Table 4), when body weight, length and head circumference were similar among the three groups of infants (Table 4). The final blood sample was drawn after four further weeks of feeding and during the fifth week of life (Table 4). By the fifth week, formula fed infants exhibited greater rates of growth in body weight than infants fed EBM (Table 4). Linear growth was not apparently different between infants fed SMA-24 and LCPE-SMA.

As expected, fatty acid analyses of red blood cell phospholipids during the first and fifth week of life indicated few differences between dietary treatment and age in fatty acid composition (data not shown). We found it difficult to conclude how the fatty acid composition of the red blood cell membrane phospholipids reflects diet and the infant's overall essential fatty acid status during this period of life.

Although the 18:2ω6 content of SMA-24 is similar to that found in human milk, a higher content of 18:2ω6 was found in plasma phospholipids of infants fed SMA-24 than in plasma phospholipids of milk-fed infants (Table 5). When the fatty acid profile of SMA was modified by incorporation of long-chain polyenoic essential fatty acids, the high level of 18:2ω6 in plasma phospholipid fraction normalized to a level similar to that found in infants fed human milk. By the fifth week of life, plasma phospholipid levels of C_{20} and C_{22} ω6 and ω3 fatty acids in infants fed LCPE-SMA were identical to those levels found in infants fed EBM, which are significantly higher than levels characteristic of infants fed SMA-24. Feeding EBM and LCPE-SMA with a balance in C_{20} and C_{22} ω6 and ω3 fatty acids resulted in similar incorporation of these fatty acids into phospholipids derived from liver or perhaps from the small intestine.

Feeding formula with a balance in long-chain polyenoic essential fatty acid similar to that of human milk (LCPE-SMA) can result in synthesis of major plasma lipid fractions with similar fatty acid content to that observed for infants fed human milk. Based on the plasma phospholipid content of C_{22} and C_{22} ω6 and ω3 fatty acids, it is apparent that the small intestine and liver of infants fed EBM or LCPE-SMA produce lipids of fundamentally different composition as compared to those of infants fed SMA-24. Whether or not tissues, such as developing brain and retina, utilize essential fatty acid constituents derived from the plasma compartment and the liver for structural membrane synthesis in a manner that also reflects similar, clearly diet-induced differences in fatty acid composition remains to be determined. Animal studies indicate that dietary intake, which produces physiological change in membrane structure and function in the intestinal mucosa and liver (22,23), also results in differences in membrane composition and transitions in functions in other tissues, including specific brain

TABLE 4

Subject Age, Body Weight, Length and Head Circumference At Time of Blood Sampling[a]

	First week of life			Fifth week of life		
	EBM[b]	LCPE-SMA[c]	SMA-24[d]	EBM	LCPE-SMA	SMA-24
Age (d)	5.1 ± 2.2	4.8 ± 2.2	5.6 ± 2.2	32.5 ± 4.8	34.2 ± 2.93	33.3 ± 8.6
Body weight (g)	1120 ± 238	1300 ± 169	1260 ± 240	1530 ± 320	2130 ± 247	2040 ± 345
Crown-heel length (cm)	37.1 ± 2.7	39.5 ± 1.9	38.9 ± 2.1	40.6 ± 2.4	45.1 ± 2.3	43.5 ± 2.2
Head circumference (cm)	25.7 ± 1.7	28.1 ± 2.9	27.2 ± 2.6	28.8 ± 2.0	31.1 ± 1.7	30.6 ± 1.9

[a]Values represent the mean ± SD.
[b]As in Table 2.
[c]As in Table 2.
[d]As in Table 2.

TABLE 5

Fatty Acid Content of Infant Plasma Phospholipid Fraction[a]

	First week of life			Fifth week of life		
	EBM[b]	LCPE-SMA[c]	SMA-24[d]	EBM	LCPE-SMA	SMA-24
			(μg/mL plasma)			
18:2ω6	138 ± 27.5	200 ± 36.9	348 ± 51.9	270 ± 3.5	285 ± 26.4	435 ± 34.8[e]
20:4ω6	181 ± 13.9	136 ± 11.0	131 ± 14.1	140 ± 40.4	102 ± 10.7	82.2 ± 9.0[e]
20:5ω3	5.1 ± 0.9	5.1 ± 1.2	3.5 ± 0.8	8.2 ± 3.8	13.2 ± 1.2	3.6 ± 0.6[e]
22:6ω3	37.8 ± 4.5	30.8 ± 3.5	21.4 ± 5.9	40.8 ± 3.2	38.0 ± 2.5	13.2 ± 2.3[f]

[a]Values represent the mean ± SD.
[b]As in Table 2.
[c]As in Table 2.
[d]As in Table 2.
[e]Significantly different from EBM and LCPE-SMA groups after four weeks of feeding, $P < 0.001$.
[f]Significantly different from EBM and LCPE-SMA groups after four weeks of feeding, $P < 0.01$.

membranes (for reviews, see refs. 2, 3, 22). Moreover, dietary fat composition has clear effects on intestinal desaturase activity (24). Whether or not such differences in function can be demonstrated for infants and are beneficial remains to be determined.

Several parameters need to be evaluated to fully evaluate if the premature infant requires nutritional support with C_{20} and C_{22} ω6 and ω3 fatty acids. First, it is clear from presently available data that feeding *vs.* not feeding very long-chain polyenoic essential fatty acids results in differences in the composition of a variety of fatty acid pools potentially available for uptake by tissues. Recent research in our laboratory indicates that the enterocyte in the brush border has desaturase activity (23) that responds by adaptation to physiological state and short-term change in diet fat intake (24). Secondly, the role of enterocyte Δ6, Δ5 and Δ4 desaturase activity in the infant's small bowel in meeting the infant's requirements for C_{20} and C_{22} ω6 and ω3 fatty acids must be examined. It is conceivable that although neural tissues may not synthesize the required C_{20} and C_{22} homologues of 18:2ω6 and 18:2ω3, the enterocyte may at some stage of life have sufficient capability to supply required chain elongated desaturated essential fatty acids. Based on differences in C_{20} and C_{22} ω6 content of plasma phospholipids between milk and formula fed infants, this stage must occur after five weeks of life. Third, definition of fatty acid pools and their dynamic turnover for uptake into neural and excitable tissues should be determined to enable provision of enrichment of C_{20} and C_{22} ω6 and ω3 constituents into the most appropriate pool for uptake. The relationship between functional tests and structural membrane composition is likely to remain tenuous as it is impossible to biopsy critical tissues. This relationship will need to be more closely established before meaningful functional assessments can be associated with differences in nutritional intake. Moreover, animal studies, while helpful, can never provide the final answer to the requirements of the preterm infant. Finally, this assessment largely ignores the potential impact of diet fat composition on the process of myelin formation in brain. Clearly, before the optimum fat composition of infant formulas can be defined, it will be necessary to determine how the level and balance between ω6 and ω3 fatty acids alters incorporation of saturated and monounsaturated fatty acids into myelin. Feeding infants LCPE-SMA results in an overall balance between C_{20} and C_{22} ω6 to ω3 fatty acids more similar to that characteristic of infants fed human milk, as illustrated by lowering 18:2ω6 and raising 20:4ω3 and 22:6ω3 content of the plasma phospholipids.

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First Year Growth of Preterm Infants Fed Standard Compared to Marine Oil n-3 Supplemented Formula[1]

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Very low birth weight (VLBW) infants (748–1390 g, n = 65) were randomly assigned to receive control or marine oil-supplemented formula when they achieved intakes >454 kJ (110 kcal)/kg/d of a formula designed for VLBW infants. Study formulas with or without marine oil were provided until 79 wk of postconceptional age (PCA), first in a formula designed for preterm infants followed by a formula designed for term infants. Infants were studied at regular intervals through 92 wk PCA. Weight, length, and head circumference were determined by standardized prodedures and normalized to the National Center for Health Statistics figures for growth of infants born at term of the same age and gender. Mean normalized weight, weight-to-length, and head circumference were greatest at 48 wk and decreased thereafter. The decline in normalized weight was greater in infants fed the marine oil-supplemented formula. Beginning at 40 wk, marine oil-supplemented infants compared to controls had significantly poorer Z-scores for weight, length and head circumference. In addition, birth order (negatively) and maternal height (positively) influenced weight and length achievement in infancy as shown previously in infants born at term.
Lipids 27, 901-907 (1992).

The purpose of this study was to compare growth in preterm infants who were randomly assigned to receive either commercially available formulas or experimental formulas (also prepared commercially) which included a low level of marine oil. The marine oil was added experimentally to test the hypothesis that membrane phospholipid docosahexaenoic acid (DHA, 22:6n-3) could be maintained in a physiological range (1,2) and the development of visual acuity enhanced (3) compared to controls. The experimental formulas provided 0.2% of total fatty acids as DHA, the amount reported in milk of American women delivering prematurely (1,4). The marine oil added to the experimental formulas also provided 0.3% of total fatty acids as eicosapentaenoic acid (EPA, 20:5n-3). This EPA concentration is higher than generally found in human milk (1,4) without regular fish (5) or fish oil (6) consumption. Together, DHA and EPA provided 0.5% of total fatty acids (approximately 0.25% of dietary energy) in the experimental formulas.

Two studies in rodents had shown that marine oil reduced weight gain during periods of rapid growth and development (7,8). In both of these studies, marine oil provided a much greater proportion of total energy than in the present infant study. On the other hand, the rodent studies were of only 2 to 3 wk duration whereas preterm infants were fed the marine oil-supplemented formula for many months. Consequently, we monitored growth in these infants to assure that growth was not adversely affected by the low levels of marine oil included in the experimental formulas. The results of this study show that low levels of marine oil supplementation reduced growth in very low birth weight (VLBW) preterm infants compared to controls beginning approximately 8 wk after feeding.

METHODS

Selection of patients. Infants selected for this study were admitted to the Newborn Center at the University of Tennessee, Memphis, TN. Although infants weighed between 748 and 1390 g at birth, they had no risk factors for poor growth other than prematurity, *i.e.,* they were not severely growth retarded *in utero* (weight less than the 5th percentile for their gestational age), and did not require long-term mechanical ventilation or gastrointestinal surgery after birth. Other exclusion criteria (intraventricular/periventricular hemorrhage > grade 2, retinopathy of prematurity > stage 2, history of maternal cocaine use) were designed to eliminate risk factors for cognitive and visual development. Infants developing problems after enrollment were replaced with another infant assigned to the same formula.

Study design. The study was designed to test the effects of marine oil-supplementation on biochemistry, visual acuity, cognition and growth in the first year of life. This paper deals with the effects on growth. Infants were eligible for enrollment when they had reached intakes of a nutrient enriched formula designed for preterm infants >454 kJ (110 kcal)/kg/d. Parental consent was obtained according to an Institutional Review Board-approved protocol, and infants were randomized to receive either the standard or the marine oil-supplemented formula. Infants were assigned a postconceptional age (PCA) in weeks by averaging the obstetric assessments of age (dates and ultrasound). If there was a discrepancy of >2 wk between these and the pediatric assessment of PCA based on physical and neurological maturity (9), all assessments were averaged.

Infants were considered study completers if they remained in the study through 57 wk PCA. A total of 79 patients were enrolled, ten to replace infants who did not complete the study through 57 wk for a variety of reasons. Four additional infants were excluded for receiving enteral nutrition other than study formulas through 57 wk. The profile of the 65 infants who completed the study is shown in Table 1. All 65 infants remained in the study through 68 wk. At 79 wk, 31 control and supplemented infants returned for followup. At 92 wk, 28 infants in each group returned for followup.

[1]Based on a paper presented at the Symposium on Milk Lipids held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

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Abbreviations: AGA, appropriate-for-gestational age; ANOVA, analysis of variance; BPD, bronchopulmonary dysplasia; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PCA, postconceptional age; SGA, small for gestational age; VLBW, very low birth weight.

TABLE 1

Neonatal and Perinatal Characteristics of the Study Population of Preterm Infants by Formula[a,b]

	Control (n = 34)	Marine supplemented (n = 31)
Birth weight (g)	1074 ± 193 (740-1398)	1147 ± 154 (833-1350)
Gestational age (wk)	29 ± 2 (26-32)	29 ± 2 (26-32)
Enrollment weight (g)	1304 ± 183 (1072-1730)	1330 ± 129 (1040-1652)
Enrollment age (d)	25 ± 10 (10-44)	22 ± 8 (9-40)
Log ventilator (h)	1.10 (0-2.775)	0.775 (0-2.547)
Log oxygen (h)	1.76 (0-2.919)	1.66 (0.48-3.196)
First formula fed (h)	78 ± 55 (32-310)	79 ± 54 (27-272)
I.V.[c] nutrition (d)	18 ± 9 (7-41)	18 ± 8 (8-38)
Size at birth (AGA/SGA)[d]	31/3	31/2
BPD[e] (no/yes)	33/1	30/2
Birth order (no.)	2.5 ± 1.4 (1-6)	2.3 ± 1.1 (1-6)
Maternal height (cm)	163.1 ± 7.7 (149-180)	164.8 ± 7.9 (152-184)

[a]Mean ± SD (range).

[b]Significant differences between dietary groups did not occur for any descriptor.

[c]Intravenous.

[d]Appropriate for gestational age (AGA or small for gestational age [SGA]).

[e]Bronchopulmonary dysplasia.

TABLE 2

Fatty Acid Composition[a] of Standard (control) and Experimental (marine oil-supplemented) Preterm (PT) and Term (T) Formulas

	Preterm formulas		Term formula	
Fatty acid	Control-PT	Experimental-PT	Control-T	Experimental-T
	g/100 g total fatty acid			
6:0	0.3	0.3	0.1	0.1
8:0	28.8	28.8	2.3	2.2
10:0	14.5	14.2	2.0	2.0
12:0	10.0	9.7	17.2	16.9
14:0	4.3	4.2	7.2	7.2
16:0	6.3	6.2	9.9	10.0
16:1	0.2	0.3	n.d.[b]	n.d.
17:0	0.1	0.1	n.d.	n.d.
18:0	3.1	3.0	4.7	4.6
18:1	9.9	9.8	17.1	17.0
18:2n-6	19.1	18.7	33.2	32.6
20:0	0.3	0.3	0.6	0.6
18:3n-3	3.0	3.1	4.8	4.9
22:0	0.2	0.2	0.3	0.3
20:5n-3	n.d.	0.3	n.d.	0.3
24:0	0.1	0.1	0.1	0.1
22:6n-3	n.d.	0.2	n.d.	0.2
C_{18} n-6/n-3	6.4	6.0	6.9	6.6
$\geq C_{20}$ n-3	0	0.5	0	0.5

[a]Provided by Ross Laboratories, Columbus, OH. [b]Not detected.

From enrollment until discharge (1800 g) infants were fed a preterm formula with or without marine oil supplementation. From discharge until 79 wk, infants received a formula designed for term infants with or without marine oil supplementation according to their original assignment. The fatty acid composition of control and experimental preterm and term formulas is shown in Table 2. Formula A and A-T were commercially available; Formula B and B-T were experimental.

Infants were studied at the following ages adjusted for early delivery: term (38 ± 2 wk PCA, mean ± SD), 2 mon (48 ± 2 wk), 4 mon (57 ± 2 wk), 6.5 mon (68 ± 2 wk), 9 mon (79 ± 2 wk) and 12 mon (93 ± 2 wk). Nutrition counseling at each visit emphasized methods for assuring that infants were fed as much formula as desired without overfeeding or underfeeding. A history of dietary intake was obtained. All except one infant consumed at least 0.72 L of formula per day through 79 wk PCA.

Reported mean energy intakes from formula (kcal/day; kcal/kg/d) were not affected by dietary assignment or gender at 48 and 57 wk PCA. At 68 wk PCA, infants consuming the marine oil-supplemented formula had significantly higher energy intakes from formula (kcal/day; kcal/kg/d) compared to infants fed standard formula (Table 3).

Measurement of growth. Infants were weighed undressed on a calibrated infant balance. Length was determined in the supine position to the nearest 0.1 cm by two individuals using an infant measuring board (Jim's Instrument Manufacturing, Inc., Coralville, IA). Head circumference was measured at the largest occipitofrontal circumference with a non-stretchable paper tape. Individual size determinations were made at each age and group data expressed as mean ± SD for a given PCA with the exception of 40 wk PCA which was interpolated from sizes at the 38 and 48 wk measurements. The growth of individual preterm infants was also normalized to that of term infants of the same gender and precise age (10) using data from the National Center for Health Statistics (Hyattsville, MD) (11) to generate percentiles for norms. The growth index generated was an approximate Z-score which indicated the SD from the fiftieth percentile (+ or −).

Statistical methods. The effects of formula on normalized first year growth were evaluated by a repeated measures analysis of variance (ANOVA) for the effects of time and marine oil supplementation (Fig. 1). A similar analysis was done for each diet-gender group to compare absolute measures of size, but this data should be interpreted with caution since the numbers of infants in each diet-gender group are not adequate to eliminate a Type II error. Preplanned comparisons between least-squares means were made using Fisher's Least Significant Difference (12). Stepwise multiple linear regression was used to determine the independent factors which affect normalized growth at each age. In addition to marine oil-supplementation (shown here to affect growth), maternal height and birth order (shown previously to affect growth in term infants, 13) were included. All statistical analyses used software from SAS Institute Inc. (Cary, NC) (14) and the mainframe computer at University of Tennessee.

RESULTS

Population of infants studied. Thirty-four control and 31 marine oil-supplemented infants completed the study. The groups were statistically similar with regard to all descriptors (Table 1) and were not at risk for poor growth. Infants with chronic lung disease (bronchopulmonary dysplasia, BPD) are known to have poorer growth (15–17). Although three of the final 65 infants were diagnosed with BPD, all of these infants were weaned from oxygen before discharge and had growth indistinguishable from their dietary group. Intrauterine growth retarded infants are also known to have poorer growth achievement in the first year of life (18). Most of the infants who completed the study (= 60) were appropriately grown (>10th percentile for gestational age), and the remaining five infants were mildly growth retarded at birth (*i.e.,* between the 5th and 10th percentile.

Weight achievement. At enrollment, all diet-gender groups had the same absolute weight (1.3 ± 0.13 to 0.18 kg, mean ± SD) (Table 4). Likewise, at the term visit (38 ± 2 wk PCA), diet-gender differences were not seen: male control 2.25 ± 0.21 kg; male marine oil-supplemented, 2.44 ± 0.41 kg; female control, 2.42 ± 0.34 kg; female marine oil-supplemented, 2.28 ± 0.20 kg (mean ± SD). However, by 40 wk, and continuing throughout infancy, infants supplemented with marine oil had lower normalized weight than those fed standard formula by Fisher's Least Squares Difference (Fig. 1A). The Z-scores depicted in Figure 1A are the most valid measure of comparison as they are adjusted for gender differences, making the number of infants per group large enough to avoid Type II errors. In addition, the Z-score is calculated for the precise age of each infant at the time of growth assessment. Although absolute and normalized weight by age, diet and gender are included in Table 4 as a basis for comparison with the data of others, they do not meet these criteria and a null hypothesis is not necessarily correct.

Length achievement. Normalized lengths were significantly lower in marine oil-supplemented infants compared to controls by 40 wk PCA ($P < 0.05$) and remained lower ($P < 0.01$) for the remainder of infancy (Fig. 1B). As with weight, the only truly valid comparison between formulas is seen in Figure 1B. The mean absolute and normalized values for length by formula, time and gender are shown in Table 5 for comparison with the data of others. Infants returning for their term visit (38 ± 2 wk PCA) were 44.1 ± 1.3, 45.6 ± 1.8, 45.4 ± 1.8 and 44.5 ± 1.0 cm long (mean ± SD; male control, male marine, female control and female marine, respectively).

TABLE 3

Mean Reported Energy Intake from Formula of Infants Fed Standard (control) and Marine Oil-Supplemented Formula at 48, 57 and 68 Wk Postconceptional Age

		48 Wk		57 Wk		68 Wk	
Formula	Gender	kcal	kcal/kg/d	kcal	kcal/kg/d	kcal	kcal/kg/d
Control	male	718	150	759	112	627	80
	female	599	129	649	108	650	90
Marine	male	599	126	712	113	764	104
	female	592	138	692	127	712	108
ANOVA[a]	formula	n.s.[b]	n.s.	n.s.	n.s.	$P < 0.04$	$P < 0.01$
	gender	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a]Analysis of variance. [b]Not significant ($P > 0.05$).

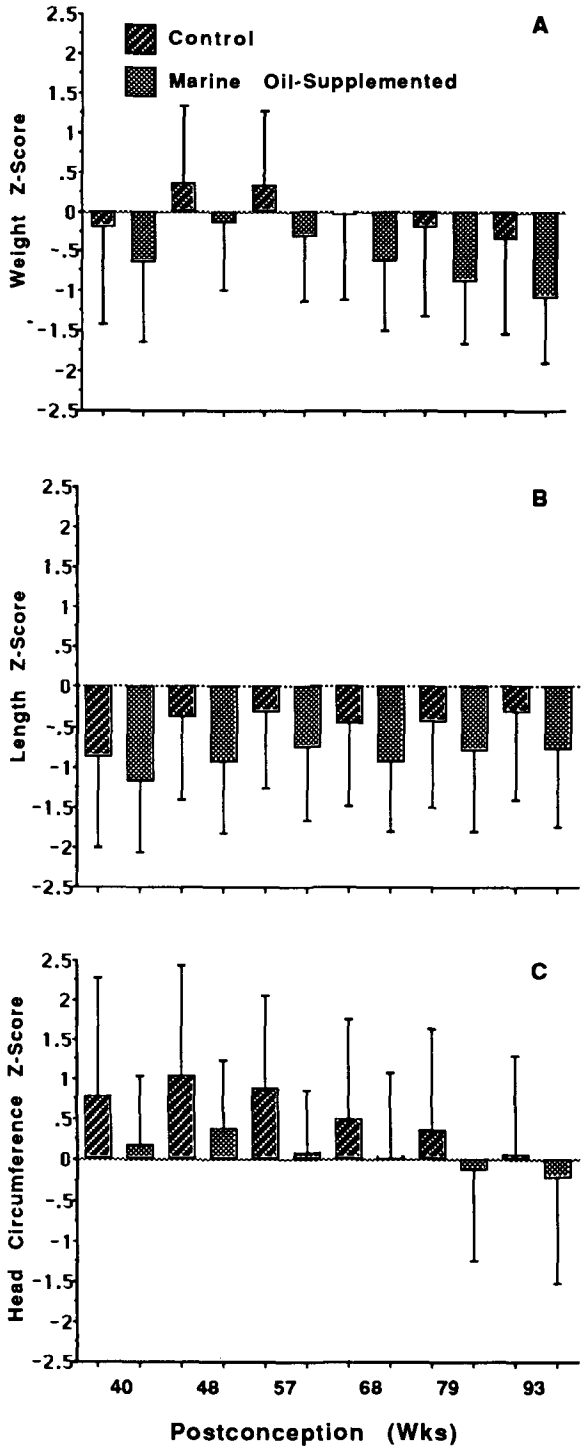

FIG. 1. Normalized growth of control and marine oil-supplemented infants at regular intervals from 40 to 93 wk postconception (mean ± SD). Individual comparisons were made between formulas using Fisher's Least Squares Difference (12). Control infants fed commercially available formulas were larger than marine oil-supplemented infants at all ages beginning at 40 wk ($P < 0.01$). The only exceptions were length at 40 wk and head circumference at 93 wk which were significant at $P < 0.05$. Analysis of variance of Z-score by formula and time: A. Weight: Formula, $P < 0.04$; time, $P < 0.001$; formula by time, n.s.; B. Length: Formula, $P < 0.08$; time, $P < 0.001$; formula by time, n.s.; C. Head circumference: Formula, $P < 0.056$; time, $P < 0.001$; formula by time, n.s.

Weight-to-length ratio. Independent of formula, the normalized weight-to-length ratio decreased significantly over time ($P < 0.0001$, Table 6). Marine oil supplementation did not affect weight-to-length ratios by ANOVA, but individual means for marine oil-supplemented infants at 79 and 93 wk PCA were lower than in the controls ($P < 0.01$). These data are not shown.

Head circumference. Head circumference Z-scores were significantly influenced by time and marine oil supplementation. Z-scores declined over time and were significantly lower in infants fed the experimental formulas compared to the standard formulas at all ages (Fig. 1C). Head circumference was the only measurement in which gender differences were seen after data were normalized to the size of term infants of the same age and gender: males had a normalized head circumference smaller than that of females at 68, 79 and 93 wk PCA (ANOVA by gender and formula, $P < 0.01$). Absolute and normalized values for head circumference by formula, time and gender are given in Table 7. As with absolute measurements of weight and length, these values are presented primarily for purposes of comparison with the data of others.

Factors affecting growth achievement. Marine oil supplementation negatively affected first year growth in these preterm infants. Previously, maternal height and birth order have been shown to affect first year growth of term infants (13). The effects of these independent variables (maternal height, birth order and marine oil supplementation) on first year growth were studied by stepwise multiple regression. Length was positively associated with maternal height, but negatively associated with marine oil supplementation; weight was negatively associated with both birth order and marine oil supplementation (Table 8).

DISCUSSION

Studies of VLBW infants cared for in neonatal intensive care centers in the late 1980s have repeatedly shown that growth in the first year of life, even when corrected for prematurity, is not equivalent to that of infants born at term. Karniski *et al.* (18) demonstrated that growth of appropriate-for-gestational age (AGA) VLBW infants appeared to be catching-up to that of infants born at term when expressed by chronological age, but not when normalized for age and gender. Georgieff *et al.* (19) also showed that VLBW infants (mean birth weight 1143 g) did not reach term-equivalent growth by 92 wk PCA, and an eight-site collaborative study of <1250 g birthweight infants concluded that normalized growth did not catch up by the 12-mon examination (20).

Infants enrolled in our study were a select group of VLBW infants without prolonged ventilator requirements or other serious illness which would limit energy intake or growth, and without evidence of chronic illness after discharge. Their parents received counseling about appropriate feeding at regular intervals during their first year at home to insure that intakes of formula were voluntary and unlimited. Growth achievement in these infants was excellent compared to published data of first year growth, *i.e.*, despite their very low birth weight (mean 1074 g), they achieved weights, lengths and head circumferences in the first year of life equivalent to much larger male and female infants (mean birth weight 1690 g)

TABLE 4

Absolute (kg) and Normalized (Z-score) Weight Achievement[a] of Very Low Birth Weight Infants in Year One by Formula and Gender

Postconceptional age (wk)	Control				Marine supplemented			
	Male (n = 11)		Female (n = 23)		Male (n = 13)		Female (n = 18)	
	kg	Z-score	kg	Z-score	kg	Z-score	kg	Z-score
33 ± 2	1.31	—	1.29	—	1.33	—	1.33	—
40	3.03	−0.61	3.13	−0.13	2.87	−0.60	2.94	−0.68[c]
48 ± 2	4.65	−0.07	4.63	0.51	4.84	−0.05	4.23[c]	−0.23[c]
57 ± 2	6.43	−0.10	6.12	0.41	6.40	−0.18	5.63[c]	−0.42[c]
68 ± 2	7.91	−0.32	7.40	−0.03	7.58	−0.46	6.77[c]	−0.73[c]
79 ± 2	9.06	−0.37	8.24	−0.24	8.21[c]	−0.84[b]	7.70[c]	−0.84[c]
93 ± 2	10.08	−0.33	9.13	−0.50	8.78[c]	−1.14[c]	8.61[c]	−0.90[c]
ANOVA[d]	Male (kg)		Male (Z-score)		Female (kg)		Female (Z-score)	
Formula	n.s.[e]		n.s.		$P < 0.02$		$P < 0.02$	
Time	$P < 0.0001$		$P < 0.0001$		$P < 0.0001$		$P < 0.0001$	
F by T	$P < 0.0001$		n.s.		n.s.		n.s.	

[a]Least-squares means for gender-formula subgroups. [b]$P < 0.05$ and [c]$P < 0.01$ compared to controls of the same gender and time by Fisher's Least Significant Difference. [d]Analysis of variance. [e]Not significant ($P < 0.05$).

TABLE 5

Absolute (cm) and Normalized (Z-score) Length Achievement[a] of Very Low Birth Weight Infants in Year One by Formula and Gender

Postconceptional age (wk)	Control				Marine supplemented			
	Male (n = 11)		Female (n = 23)		Male (n = 13)		Female (n = 18)	
	cm	Z-score	cm	Z-score	cm	Z-score	cm	Z-score
40	47.5	−1.09	48.1	−0.83	47.6	−1.06	47.1[b]	−1.27[c]
48 ± 2	53.7	−0.83	54.6	−0.25	54.8[b]	−0.88	53.0[c]	−0.99[c]
57 ± 2	60.9	−0.70	60.4	−0.21	61.3	−0.72	59.4[b]	−0.79[c]
68 ± 2	66.7	−0.62	65.6	−0.39	65.6[b]	−1.05[b]	64.1[c]	−0.89[c]
79 ± 2	69.9	−0.84	69.4	−0.25	69.4	−0.89	68.2[c]	−0.75[c]
93 ± 2	74.4	−0.59	73.7	−0.28	73.1[b]	−0.96	72.3[c]	−0.63[b]
ANOVA[d]	Male (kg)		Male (Z-score)		Female (kg)		Female (Z-score)	
Formula	n.s.[e]		n.s.		$P < 0.08$		$P < 0.07$	
Time	$P < 0.0001$		n.s.		$P < 0.0001$		$P < 0.0001$	
F by t	$P < 0.02$		n.s.		n.s.		n.s.	

[a]Least-squares means for gender-formula subgroups. [b]$P < 0.05$ and [c]$P < 0.01$ compared to controls of the same gender and time by Fisher's Least Significant Difference. [d]Analysis of variance. [e]Not significant, or with a trend toward significance ($P > 0.10$).

TABLE 6

Normalized (Z-score) Weight-to-Length Ratios[a] of Very Low Birth Weight Infants in Year One by Formula and Gender

Postconceptional age (wk)	Control		Marine supplemented	
	Male (n = 11)	Female (n = 23)	Male (n = 13)	Female (n = 18)
	Z-score	Z-score	Z-score	Z-score
48 ± 2	1.03	0.73	0.85	0.61
57 ± 2	0.80	0.65	0.44	0.25[c]
68 ± 2	0.39	0.37	0.38	−0.03[b]
79 ± 2	0.60	0.07	−0.15[c]	−0.21
93 ± 2	0.46	−0.16	−0.60[c]	−0.48[b]
ANOVA[d]	Male (Z-score)		Female (Z-score)	
Formula	n.s.[e]		n.s.	
Time	$P < 0.0001$		$P < 0.0001$	
F by T	n.s.		n.s.	

[a]Least-squares means for gender-formula subgroups. [b]$P < 0.05$ and [c]$P < 0.01$ compared to controls of the same gender and time by Fisher's Least Significant Difference. [d]Analysis of variance. [e]Not significant ($P > 0.05$).

TABLE 7

Absolute (kg) and Normalized (Z-score) Head (occipitofrontal) Circumference Ratios[a] of Very Low Birth Weight Infants in Year One by Formula and Gender

Postconceptional age (wk)	Control				Marine supplemented			
	Male (n = 11)		Female (n = 23)		Male (n = 13)		Female (n = 18)	
	cm	Z-score	cm	Z-score	cm	Z-score	cm	Z-score
40	35.0	0.05	35.3	1.13	34.7	0.18	35.0	0.20[c]
48 ± 2	38.6	0.33	38.9	1.38	39.2[b]	0.41	37.9[c]	0.39[c]
57 ± 2	41.8	0.06	41.8	1.16	41.8	0.06	40.7[c]	0.12[c]
68 ± 2	44.3	−0.03	43.7	0.76	43.9	−0.06	42.9[c]	0.10[c]
79 ± 2	45.7	−0.09	45.0	0.58	45.1[b]	−0.32	44.3[b]	−0.02[c]
93 ± 2	46.8	−0.26	46.0	−0.25	46.1[b]	−0.55	45.7	0.00

ANOVA[d]	Male (cm)	Male (Z-score)	Female (cm)	Female (Z-score)
Formula	n.s.[e]	n.s.	$P < 0.07$	$P < 0.03$
Time	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
F by T	$P < 0.04$	n.s.	n.s.	$P < 0.01$

[a]Least-squares means for gender-formula subgroups. [b]$P < 0.05$ and [c]$P < 0.01$ compared to controls of the same gender and time by Fisher's Least Significant Difference. [d]Analysis of variance. [e]Not significant or with a trend toward significance ($P > 0.10$).

TABLE 8

Normalized Growth Achievement in the First Year of Life. Multiple Regression Analysis Included Birth Weight (forced),[a] Maternal Height, Birth Order and Marine Oil Supplementation (marine oil)

Dependent variable	Age[b]	Independent variable	F	Probability	Model r^2[c]
Weight	40	marine oil (−)[d]	4.73	0.03	0.13
	48	marine oil (−)	14.13	0.001	
		maternal height (+)	6.19	0.02	0.28
	57	marine oil (−)	15.43	0.001	
		birth order (−)	8.12	0.006	0.33
	68	marine oil (−)	12.88	0.001	
		birth order (−)	7.17	0.01	0.30
	79	marine oil (−)	10.28	0.002	0.17
	93	marine oil (−)	8.84	0.005	
		birth order (−)	4.48	0.04	0.24
Length	40	no variable met the $P < 0.05$ level of significance			
	48	maternal height (+)	9.26	0.004	
		marine oil (−)	11.87	0.001	0.29
	57	maternal height (+)	13.21	0.001	
		marine oil (−)	10.07	0.003	
		birth order (−)	4.73	0.03	0.36
	68	marine oil (−)	10.69	0.002	
		maternal height (+)	7.89	0.007	
		birth order (−)	8.55	0.005	0.35
	79	maternal height (+)	10.16	0.003	
		marine oil (−)	6.70	0.01	0.22
	93	maternal height (+)	13.71	0.001	
		marine oil (−)	7.24	0.01	0.28
Head circumference	40	marine oil (−)	4.15	0.05	0.08
	48	marine oil (−)	6.33	0.02	0.12
	57	marine oil (−)	9.40	0.01	0.16
	68	marine oil (−)	4.13	0.05	0.07
	79	no variable met the $P < 0.05$ level for entry into the model			
	93	no variable met the $P < 0.05$ level for entry into the model			

[a]Birth weight was forced into each analysis, but did not reach significance for any dependent variables.
[b]Postconceptional age (wk).
[c]Proportion of the variance predicted by the significant independent variables.
[d]The sign indicates if the association with height is negative (−) or positive (+).

in an eight-center survey (20). Nevertheless, infants supplemented with marine oil compared to controls had significantly lower Z-scores for weight and length by 40 wk PCA; these lower Z-scores continued throughout the first year of life. The lower normalized growth in marine oil-supplemented infants was not related to lower energy intakes compared to controls (Table 3).

Infants fed both standard and marine oil-supplemented formulas achieved weights closest to standards for infants born at term when they were 48 and 57 wk PCA. After this age, their normalized weights fell consistently to the end of infancy as has been reported by others (18,19). The decline in weight in late infancy was greater in marine oil-supplemented infants compared to those infants fed standard formulas, and appeared to be responsible for the significantly lower weight-to-length ratio seen in the supplemented compared to the control infants at 79 and 93 wk PCA.

The mechanisms by which growth of VLBW infants falls progressively farther behind that of term infants in the last half of infancy is not known. This pattern of growth is more consistent with a response to some extrinsic factor, *e.g.*, the quality and quantity of nutritional intake, than with failure to recover from the insults of the neonatal intensive care period. The mechanism by which such low levels of marine oil supplementation might influence growth in preterm infants is also not known. Marine oil has been reported to slow weight gain in rodents during periods of rapid growth (7,8). Although these rodent studies were of only two to three weeks duration, they provided more dietary energy from EPA and DHA than the 0.25% of energy fed to preterm infants in this study. We recently reported a positive correlation between arachidonic acid status and growth in VLBW infants fed standard formulas (21). Since marine oil supplementation decreased the concentration of plasma arachidonic acid further (2), it is tempting to speculate that a common mechanism related to arachidonic acid status is involved in both the decline in normalized growth of preterm infants in the last half of infancy (18,19) and the greater decline in normalized growth in infants randomized here to receive marine oil-supplemented formula compared to standard formula.

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Human Mammary Gland Function at the Onset of Lactation: Medium-Chain Fatty Acid Synthesis[1]

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The onset of medium-chain fatty acid synthesis in the human mammary gland was investigated. Colostrum and serum were collected from 31 healthy women and the fatty acid composition of total lipid was analyzed by gas-liquid chromatography. Although colostrum/serum ratios for most fatty acids range from 0.7–2.4, very low levels of 10:0 and 12:0 were present in serum lipids as compared to much higher concentrations of these fatty acids in colostrum lipids (colostrum/serum ratio 16.23 and 17.11 for 10:0 and 12:0, respectively). We have previously found that medium-chain fatty acid levels are very low in *prepartum* mammary secretions (6–10 wk before term delivery) but are higher and similar in colostrum of women who deliver preterm (3–14 wk) or at full term. The data indicate that parturition, irrespective of length of pregnancy, is the trigger for medium-chain fatty acid synthesis in the human mammary gland.

Lipids 27, 908–911 (1992).

Milk fat is important for the newborn infant as a major source of energy and as a source of specific fatty acids that are essential for normal growth and development (1). Long-chain polyunsaturated fatty acids are essential for brain development (2), myelination (3) and retinal function (4) and medium-chain fatty acids are important as an easily absorbable energy source (5). Medium-chain fatty acids can be absorbed directly through the gastric mucosa (6–8) and are transported from the intestine to the liver, where they are rapidly metabolized to ketone bodies (9). This provides a distinct advantage to the newborn. The preferential incorporation of ketone bodies into brain and lung lipids in the neonatal period (10) suggests that medium-chain fatty acids also might be important as substrates for organ growth and not only as energy source. This is consistent with reports that although these fatty acids contribute little to lipogenesis (11), they can be stored in the adipose tissue of orally fed infants (12).

Animal studies have shown that medium-chain fatty acids are synthesized within the mammary gland (13–15), contrary to the majority of long-chain fatty acids that are provided to the mammary gland through lipoprotein lipase mediated uptake from circulating lipoprotein triglyceride (16). In this study we have investigated the onset of medium-chain fatty acid synthesis in the human mammary gland.

MATERIALS AND METHODS

The subjects of this study were 31 healthy mothers of full term infants. All women gave informed consent for this study which was approved by the Institutional Review Board of the Medical Center of Delaware. The women were studied during the first two days after the delivery of healthy infants. Maternal blood specimens were taken simultaneously with the collection of colostrum. Colostrum was collected at mid-feed, as described previously (17). Serum and colostrum specimens were stored at −70°C until analysis. Lipids were extracted with chloroform/methanol (2:1, vol/vol) and the fatty acid methyl esters were prepared and quantitated by wide bore capillary gas-liquid chromatography as previously described in detail (18).

RESULTS

Data on the subject population are given in Table 1. The 31 healthy women studied consumed balanced diets resulting in a weight gain during pregnancy that is in accordance with the recent recommendation of the National Academy of Science (19). Eighty percent of the women studied were multipara, a majority of them having breastfed previously.

Analysis of the fatty acid composition of colostrum shows that most fatty acids are present at concentrations similar to those previously reported (18) (Table 2). Comparison of the composition of colostrum and serum shows similar concentrations for most long-chain fatty acids, resulting in a milk/serum ratio of 0.74 to 2.45. Exceptions were medium-chain fatty acids, present at much higher concentrations in colostrum than in serum (milk/serum fatty acid ratio as high as 17.11) and a few long-chain unsaturated fatty acids such as 20:1, 20:4n-3 and 22:5n-3 (milk/serum ratio 6.01, 2.90 and 3.87, respectively). Assuming that a ratio close to 1.0 indicates uptake of the fatty acid from the circulation after its release from chylomicrons or from very low density lipoproteins by lipoprotein lipase, at the capillary endothelium of the lactating mammary gland, a milk/serum ratio greater than 2.0 would indicate synthesis within the mammary gland. The

TABLE 1

Characteristics of the Study Population[a]

Number of subjects	31	
Age (years)	29.2 ± 0.80	(18–39)
Weight gain during pregnancy (kg)	12.60 ± 0.52	(6.8–20.9)
Colostrum lipids (g/dL)	1.59 ± 0.39	(0.23–3.36)
Serum lipids (g/dL)	0.35 ± 0.01	(0.17–0.53)
Parity	Primipara 6, multipara 25	
Previous breastfeeding	23 (92% of multipara)	

[a]Data are mean ± SEM and (range). All subjects consumed balanced diets and had no unusual food preferences.

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TABLE 2

Fatty Acid Composition of Colostrum and Serum After Full Term Delivery[a]

Fatty acid	Colostrum[b]	Serum[b]	Milk/serum ratio
Medium-chain			
8:0	0.05 ± 0.01	0.02 ± 0.01	3.18
10:0	0.32 ± 0.04	0.02 ± 0.00	16.23
12:0	2.53 ± 0.20	0.15 ± 0.02	17.11
14:0	5.68 ± 0.25	1.31 ± 0.06	4.35
Long-chain saturated			
15:0	0.36 ± 0.01	0.28 ± 0.01	1.35
16:0	25.91 ± 0.32	27.75 ± 0.36	0.93
17:0	0.45 ± 0.01	0.33 ± 0.01	1.38
18:0	7.82 ± 0.20	5.82 ± 0.13	1.35
20:0	0.29 ± 0.01	0.12 ± 0.02	2.45
Long-chain monounsaturated			
16:1	2.13 ± 0.10	2.88 ± 0.12	0.74
17:1	0.27 ± 0.01	0.29 ± 0.01	0.92
18:1	32.22 ± 0.42	22.12 ± 0.39	1.59
20:1	1.03 ± 0.04	0.17 ± 0.02	6.01
Long-chain polyunsaturated			
n-3			
18:3	0.76 ± 0.04	0.56 ± 0.02	1.35
20:3	0.06 ± 0.01	0.00	—
20:4	0.03 ± 0.01	0.01 ± 0.00	2.90
20:5	0.00	0.06 ± 0.02	—
22:5	0.15 ± 0.03	0.04 ± 0.01	3.87
22:6	0.32 ± 0.04	1.80 ± 0.05	0.18
n-6			
18:2	13.73 ± 0.83	27.14 ± 0.46	0.51
20:3	0.80 ± 0.04	1.63 ± 0.08	0.49
20:4	1.20 ± 0.05	6.79 ± 0.20	0.18
22:4	0.63 ± 0.05	0.33 ± 0.05	1.94
22:5	0.06 ± 0.02	0.32 ± 0.05	0.18

[a]Data are from 31 healthy women who delivered full term infants. The characteristics of the women studied are given in Table 1. There were no statistically significant differences in colostrum fatty acid composition as function of weight gain during pregnancy or parity, except that myristic acid (14:0) concentration was significantly higher in primipara than in multipara (6.60 ± 0.38 *vs.* 5.19 ± 0.46% $P < 0.05$).

[b]Percent of total fatty acids.

lowest colostrum/serum ratio was for arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acids, at 0.18 each. There was no difference in colostrum fatty acid composition as a function of weight gain during pregnancy or parity, except that myristic acid concentration was higher in milk lipids of primipara ($P < 0.05$).

DISCUSSION

The data presented indicate that the human mammary gland is able to synthesize *de novo* medium-chain fatty acids as well as to elongate and desaturate some long-chain fatty acids shortly after parturition. We have reported earlier that *prepartum* mammary secretions, collected between 1 and 70 days before full term delivery, contain lower amounts of fat than colostrum and mature milk (1.15 and 1.28 g/dL at an average of 42 and 9 d before delivery, respectively) and have a markedly different lipid composition than *postpartum* secretions (17). The data presented in Table 3 show that *prepartum* secretions contain considerably lower concentrations of 10, 12 and 14 than *postpartum* colostrum or mature milk (Tables 2 and 3). As *prepartum* secretions collected at an average of 42 or 70 d before term delivery correspond to secretions collected at 6–10 wk *prepartum*, we also have compared the medium-chain fatty acid composition of total lipids in these secretions with those in the colostrum secreted by women who delivered at similar periods of prematurity. As can be seen from the data presented in Table 3 (18), there was no difference in the medium-chain fatty acid level between the colostrum of women who delivered prematurely and colostrum of women who delivered after a full term pregnancy (Tables 2 and 3). Thus, parturition, irrespective of length of pregnancy, seems to be the trigger for the increase in *de novo* fatty acid synthesis within the mammary gland. It is also possible that milk removal, whether by the newborn's suckling or by milk expression, after full term or premature delivery, respectively, is an important factor in the increase of lipogenesis within the human mammary gland. Animal studies have shown that a marked increase in lipogenic enzymes acetyl-CoA carboxylase, ATP citratelyase and fatty acid synthetase occurs immediately after parturition and that milk removal is essential for this increase (20). Whether the presence of long-chain fatty acyl-CoA, which is a potent inhibitor of the two former enzymes in the mammary gland (21,22), or other factors (23) might contribute to the lower concentration of medium-chain fatty acids in *prepartum*

TABLE 3

Medium-Chain Fatty Acids in Human Mammary *Prepartum* Secretion, in Colostrum and in Milk Secreted After Premature or Full Term Delivery

Specimen	*n*	Fatty acid %		
		10:0	12:0	14:0
Prepartum secretion[a]	10	0.10 ± 0	1.7 ± 0.4	4.9 ± 0.6
Postpartum colostrum[b]				
Premature delivery				
26-30 wk	15	0.26 ± 0.14	3.09 ± 0.59	5.52 ± 0.68
31-37 wk	23	0.31 ± 0.10	3.14 ± 0.43	5.87 ± 0.50
Full term delivery	6	0.27 ± 0.22	3.10 ± 0.91	6.81 ± 1.06
Mature milk[c]				
Prepartum delivery				
26-30 wk	15	1.37 ± 0.17	7.47 ± 0.72	8.41 ± 0.83
31-37 wk	23	1.27 ± 0.18	6.55 ± 0.77	7.55 ± 0.89
Full term delivery	6	0.97 ± 0.28	4.46 ± 1.17	5.68 ± 1.36

[a]*Prepartum* secretion collected from women 1-70 d before full term delivery, from Bitman *et al.* (17).

[b]Data for colostrum and milk fatty acid composition after preterm and full term delivery, from Bitman *et al.* (18).

[c]Mature milk collected at 6 wk lactation, from Bitman *et al.* (18).

secretions is not known. The absence of lipoprotein lipase from *prepartum* secretions (17) suggests low activity in the mammary gland before term delivery, as previously shown in the rat (24). This would indicate that the long-chain fatty acids present in these secretions might originate mainly from the albumin-bound free fatty acids in the circulation. The absence of tight junctions between mammary epithelial cells before parturition would facilitate this transfer of fatty acids into the mammary gland.

Synthesis of medium-chain fatty acids is specific to the mammary gland, because only this tissue contains a specific enzyme, thioesterase II, which terminates chain elongation at 6-14 carbon atoms (25,26). In all other tissues, lipogenesis results in long-chain fatty acids associated with the enzyme thioesterase I, which terminates chain elongation at or above 16 carbon atoms (27). We could therefore use the milk/serum fatty acid ratio to assess the origin of milk fatty acids. Indeed, the serum concentration of fatty acids shorter than C_{14} was extremely low. Concentrations were, however, considerably higher in colostrum, resulting in very high milk/serum fatty acid ratios for 10:0 and 12:0, at 16.23 and 17.11, respectively.

Fatty acid synthetase has been characterized (28,29) in a permanent human mammary epithelial cell line, SkBr 3 (30), and the production of medium-chain fatty acids in *in vitro* studies has been reported (31). The onset of fatty acid synthetase and thioesterase II activity in the human mammary gland is, however, unknown. Because human lactating mammary gland cannot be studied for ethical reasons, one depends on indirect observations such as those described in this study. Based on these studies, which include colostrum (Table 3; refs. 18,32), milk (18) and serum as well as *prepartum* mammary secretion (17), one can conclude that in the human, as in other species, parturition, irrespective of length of pregnancy, is the trigger for the synthesis of medium-chain fatty acids. Whether this is dependent upon hormonal changes associated with delivery and onset of lactation or with removal of milk from the mammary gland (by suckling or pumping), or a combination of both, remains to be determined. As in other species (7), the rate of synthesis of medium-chain fatty acids increases with length of lactation (Table 3; refs. 18,33,34).

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Lactation Curves and Effect of Pup Removal on Milk Fat of C57Bl/6J Mice Fed Different Diet Fats[1]

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Groups of C57Bl/6J mice, fed either a *cis* (C-Diet) or *trans* diet (T-Diet) were milked without preconditioning at 6, 8, 10 and 12 days *postpartum*. On day 10, groups of mice were also milked 4, 6 and 18 h after separation of the pups. Except for the 18-h separation, all T-Diet fed animals produced milk of lower fat content than did the C-Diet animals ($P < 0.001$) throughout the lactation period measured. In the C-Diet mice, the 6-h separation period resulted in a decrease ($P \leq 0.03$) in fat, but the diet-depressed milk fat of T-Diet animals was not decreased further until the 18-h separation period. Milk volume increased as lactation progressed and was greatly increased as a result of preconditioning ($P \leq 0.001$), even at 4 h of separation when fat was not reduced, and was always greater for T-Diet animals. Within diet groups, fatty acid composition was similar throughout the lactation period studied and was not affected by preconditioning, except in the 18-h separation period, when *de novo* fatty acids were significantly reduced ($P \leq 0.05$). The data are consistent with the hypothesis that preconditioning results in lowered milk fat values and that preconditioning techniques can explain discrepancies in literature values for murine milk fat.
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Separation of pups from dams (preconditioning) is frequently used to increase milk volume collected from laboratory rodents (1-5). Since previous work (6), in which dams and pups were not separated prior to milking, resulted in higher milk fat values than reported for mice (7-11), it was hypothesized that the lower literature values were due to preconditioning. Thus, the major objective of the present study was to determine whether preconditioning affects the percentage of milk fat obtained from lactating mice. This paper also contains data on a partial lactation curve in the presence of suckling pups and the fatty acid composition of milk from mice fed diets with or without *trans* fatty acids.

MATERIALS AND METHODS

Diets. Upon arrival, female mice were randomly assigned to one of two diets, which were fed *ad libitum* throughout the study. The two diets were essentially identical, except for the presence or absence of *trans* fatty acids (see Tables 1 and 2). As can be seen in Table 2, nearly 50% of the 18:1 in the fat of the *trans* diet (T-Diet) consisted of *trans* isomers, whereas all of the 18:1 consisted of *cis* fatty acids in the *cis* diet (C-Diet). The T-Diet also contained less than 2% of dienes that contained one or two *trans* double bonds.

Animals. The C56Bl/6J mice used in this study were purchased as weanlings from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained under conditions conforming to the National Institute of Health guidelines (12). Mice were kept in plastic shoebox cages in a facility maintained at 23°C and 50% RH on a 12-h light cycle. Water and diet were provided *ad libitum*. At about 35 d of age, females were harem mated, with 1 male randomly assigned to each group of 4 females. After 1 wk, males were removed, each female was housed in a separate cage and births were noted daily at about noon. At day 4 *postpartum*, litter size (3-8 pups) was adjusted to 5 or 6 pups within the same diet group. Dams were allowed two days to acclimate, and milk was collected as previously described (6) on days 6, 8, 10 and 12 *postpartum*.

TABLE 1

Diet Composition

Ingredients	g/kg of Diet (dry weight)
Sucrose[a]	590
Vitamin-free casein[b]	200
Alphacel[b]	50
Fat[c]	100
AIN 76 Mineral Mix[b]	40
AIN 76 A Vitamin Mix[b]	15
DL-methionine[b]	3
Choline bitartrate[b]	2
% Energy from fat	20
% Energy as EFA[d]	5.2
% Energy as *trans* fatty acids	5.4

[a]Purchased from local grocery wholesaler.
[b]Purchased from ICN Biochemicals, Costa Mesa, CA.
[c]Defined in Table 2.
[d]EFA, essential fatty acid.

TABLE 2

Fatty Acid Composition of Diet Fat

	Fatty acid methyl ester wt%[a]	
Fatty acid[b]	C-Diet[c]	T-Diet[d]
16:0	13.4 (0.5)	10.9 (0.7)
18:0	6.5 (0.4)	9.8 (0.2)
18:1*t*	ND	25.4 (0.3)
18:1*c*	51.1 (0.7)	24.4 (0.6)
18:2n-6	26.1 (0.6)	25.6 (0.8)
18:2*i*	ND	1.5 (0.3)
18:3n-3	0.9 (0.1)	0.9 (0.1)

[a]Means ± SD of fatty acid methyl esters. Small amounts of 14:0, 16:1, 20:0, 20:1 are not listed.
[b]Fatty acid carbon number:double bonds; *c* and *t* indicate *cis* and *trans* geometric isomers. ND indicates not detected. *i*, Isomers, both *cis* and *trans*, of 18:2n-6.
[c]C-Diet (*cis*-diet) oil was a blend of olive oil (Pope olive oil, packed in Italy for Pope Food Sales, Inc., Oradell, NJ), cocoa butter (purchased from Wilbur Chocolate Company, Lititz, PA, and corn oil (Wesson corn oil, Beatrice/Hunt-Wesson, Inc., Fullerton, CA) with 0.02% w/w TBHQ (*tert*-butylhydroquinone, (Eastman Kodak Company, Rochester, NY) added as antioxidant.
[d]T-Diet oil was a blend of shortening (Bunge Shortening, manufactured by Bunge Edible Oil Corp., Kankakee, IL), corn oil (Wesson corn oil) and soybean oil (Giant brand, distributed by Giant Foods Inc., Washington, D.C.) with 0.02% TBHQ.

[1]Based on a paper presented at the Symposium on Milk Lipids held at the AOCS Annual Meeting, Baltimore, MD, April 1990.
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Abbreviations: *c*, *cis* isomers; C-Diet, *cis* fat diet; EFA, essential fatty acid; FAME, fatty acid methyl esters; ND, not detected; *t*, *trans* isomers; T-Diet, *trans* fat diet.

Lactation curve data (day 6, 8, 10 and 12) were collected from females remaining with their pups until the time of milking (1300–1500 h). After milking, they were returned to their cages and individual pup weights were obtained. Animals used in the preconditioning studies were milked after being separated from their pups for 4, 6 or 18 h. For the 4 and 6 h preconditioning data, females were separated from their pups at day 10 in the morning, at which time individual pup weights were obtained. The separated dams were milked at about 1500 h prior to reuniting them with their pups. For the 18-h separation values, pups were individually weighed and then killed at about 1700 h on day 10. The dams were milked in the early afternoon of the next day (day 11), about 18 h after separation from their pups.

A total of 25 females on the C-Diet and 20 females on the T-Diet were used in these studies. For the lactation curve studies, all mice were used for 6- and 8-day points, but 22 C-Diet and 17 T-Diet dams were used for the 12-day points. At 10 d *postpartum* the lactating females were separated into four groups. One group of 10 females on the C-Diet and 5 on the T-Diet were milked without preconditioning the dams, and the data were used in the lactation studies as well as in the preconditioning studies. A second set of dams (4 on the C-Diet and 6 on the T-Diet) were milked at 4 h after the pups were removed; a third set (5 on the C-Diet and 6 on the T-Diet) were milked 6 h after preconditioning, and the last set of 6 females, half on the C-Diet and half on the T-Diet, were milked 18 h after they were separated from their pups at day 10 *postpartum.*

Milk fat and volume measurements. The standardized creamatocrit technique for determining milk fat percentage has been described previously (6). Milk fat for fatty acid analysis was obtained by a dry column method (6,13). Milk volumes were estimated after calibrating ten of the capillary tubes (VWR, Scientific, Chicago, IL; microhematocrit) employed in the creamatocrit procedure. The capillary tubes were pre-weighed, filled about 2/3 full with distilled water, weighed again, and then sealed with a plug of Critoseal. Based on the weight and column height of the water and assuming a density of 1.0 for water, the tube volumes (average ± SD) were calculated to be 0.934 ± 0.0005 μL/mm.

Fatty acid analyses. Milk fat and dietary fats were converted to fatty acid methyl esters (FAME) using anhydrous methanolic HCl and methylene chloride as a cosolvent. After purification by thin-layer chromatography, the FAME were separated on a 25 m × 0.25 mm fused silica column coated with SP-2340 (Quadrex Corp., New Haven, CT). Procedures employed to esterify the lipids and to separate, identify and quantify the FAME have been described (14).

RESULTS AND DISCUSSION

Throughout these studies, average body weight and weight gain were similar for pups of dams fed either diet. At day 10 the C-Diet pups weighed 4.4 ± 0.4 g (mean ± SEM) and the T-Diet pups weighed 4.6 ± 0.2 g. There was no evidence that milking the dams every other day affected the health of the dams or pups or altered the milk values obtained. However, if the dams were disturbed at day 1 or 2 *postpartum*, they tended to kill the pups. By day 4 it was possible to augment or cull litters without severe losses. The addition of pups was accomplished by gently dusting the new pup with bedding from the foster mother and placing the pup under the natural pups in the cage. By day 6, it was possible to collect sufficient sample from most animals nursing 5–6 pups; however, milk volumes prior to day 6 *postpartum* were generally insufficient for analysis.

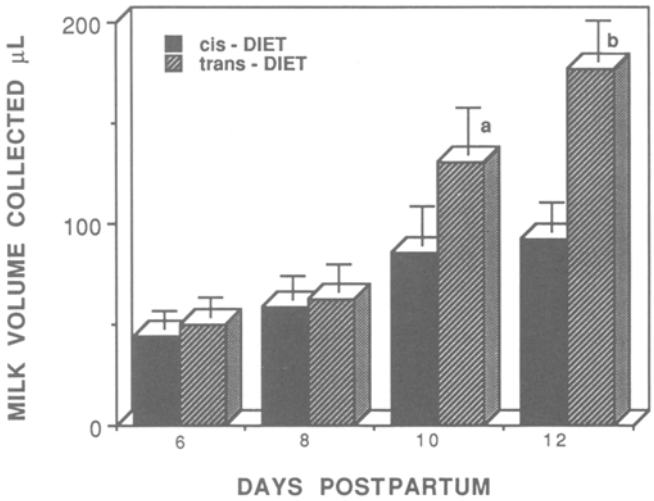

FIG. 1. Volume of milk collected as lactation progresses in C57Bl/6J mice fed the *cis* or *trans* Diet. Means and SEM are shown. For days 6, 8, 10 and 12 the number of observations for the *cis* Diet were 3, 8, 7 and 6 and for the *trans* Diet 5, 4, 5 and 5, respectively. The "a" and "b" indicate volumes significantly greater than the *cis* Diet values (*t*-test $P \leq 0.03$).

Lactation curve. Data for the lactation curve studies are shown in Figures 1 and 2. The milk volume collected (Fig. 1) increased during lactation. This was expected since growing pups require more milk. However, animals on the T-Diet always gave the larger nominal volume of milk with statistically significant differences ($P < 0.01$) at 10 and 12 days *postpartum.* At the same time, although the fat values were constant within a diet group, the dams fed the T-Diet always gave milk with fat percent values that were significantly lower ($P < 0.01$) than dams fed the C-Diet (Fig. 2).

The differences observed in the fat values were expected and confirm our previous work (6) regarding a depression of milk fat in lactating mice fed diets containing partially hydrogenated fat. In contrast, the differences in milk volume produced by dams on the two diets were not expected, but there is little doubt that they are a reflection of the differences in fat. Given the difference in fat concentration and the lack of detectable differences for either protein or lactose concentration (15), a given volume of the *trans* milk would have less calories than an equal volume of the *cis* milk. Yet, despite the lower calorie T-Diet milk, there were no significant differences in weight and growth rate of pups in the two diet groups. Thus it appears that a greater volume of the low-fat milk was required by pups in the T-Diet group to meet their caloric and nutrient needs.

Throughout these studies the volume measurements were more variable than the fat measurements. The large variability in volume may be due to the fact that pups

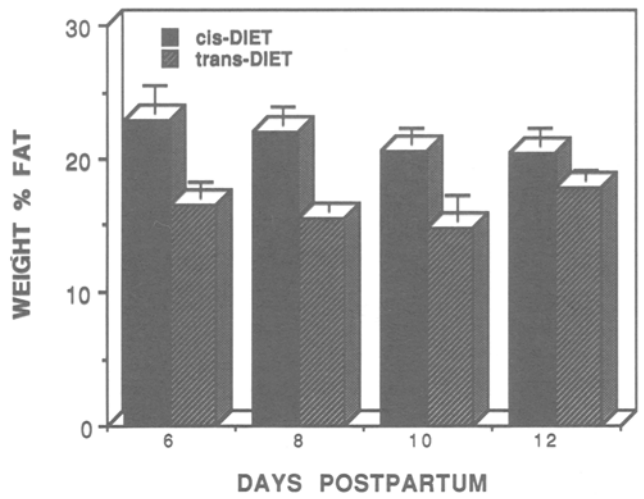

FIG. 2. Percentage milk fat from C57Bl/6J mice fed the *cis* Diet or *trans* Diet. Means and SEM are shown. All *cis* Diet values are significantly ($P \leq 0.001$) greater than all *trans* Diet values. See Figure 1 for number of observations.

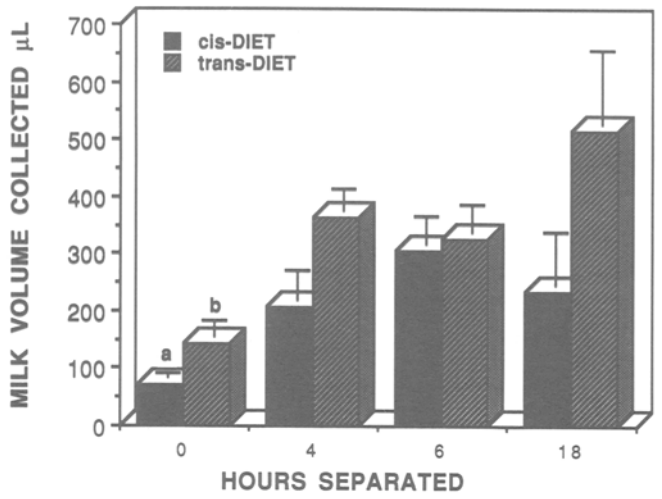

FIG. 3. Volume of milk collected from C57Bl/6J mice after separation of dams from pups. Means and SEM are shown. Sample numbers for 0, 4, 6 and 18 h are 7, 5, 5 and 3, for *cis* Diet and 5, 6, 6 and 3 for *trans* Diet, respectively. The "a" and "b" indicate significantly smaller volumes than when separated (*t*-test $P \leq 0.001$).

remained with the dams and that the time from the last nursing was not controlled. The smaller variability in the fat values was probably a reflection of the consistency of fat values obtained for "fore" and "hind" milk, as discussed previously (6).

Pup removal study. The pup removal study was conducted to test whether preconditioning practices might be responsible for discrepancies in murine milk fat values reported in the literature (6). Pups were separated on day 10 *postpartum* as lactation was clearly well established, milk volumes were greater than at earlier time points and it was likely that pups could consume milk solely at that stage of lactation. Also, 10-day-old pups were able to survive short separation times with no apparent ill effects.

As can be seen in Figure 3, milk volume was increased ($P \leq 0.001$) when pups were separated for 4 h or longer. However, preconditioning periods greater than 4 h did not yield statistically significant greater volumes of milk. Within each diet group, the fat values for these milks (Fig. 4) were not different after 4 h of preconditioning, but 6 h of preconditioning resulted in a statistically significant decrease ($P < 0.03$) in fat percent of milk from dams fed the C-Diet, and decreased fat values ($P < 0.01$) were observed for dams fed either diet after 18-h of preconditioning. It should be noted (Fig. 4) that except at the 18 h preconditioning period, fat values for dams fed the T-Diet were consistently lower ($P \leq 0.01$) than for those fed the C-Diet.

As expected, preconditioning increased milk volume, but it adversely affected the proportion of fat. The decrease in fat in the C-Diet group was a result of separation, since reuniting dams and pups resulted in normal values when checked on day 12 (data not shown). These data support the work of others who have reported that preconditioning beyond 6 h is unphysiological (11) and affects lipogenesis as well as milk secretion (16). These data also are consistent with the hypothesis that preconditioning techniques are responsible for discrepancies in milk fat values in the literature (6).

Although milk fat was decreased in C-Diet animals as a result of preconditioning at 6 h, the T-Diet animals did not respond until 18 h of separation. This difference in response may be related to the fact that T-Diet animals were already in a fat depressed state (6) (Fig. 2 and 4). It is possible that certain enzyme activities (which are sensitive to suckling) were partially depressed in animals fed the T-Diet and moderate preconditioning periods did not depress them further. It is likely that by 18 h of preconditioning, glandular involution had progressed to the point that milk synthesis was seriously impaired. Thus at this stage both diet groups gave milks with lower fat values.

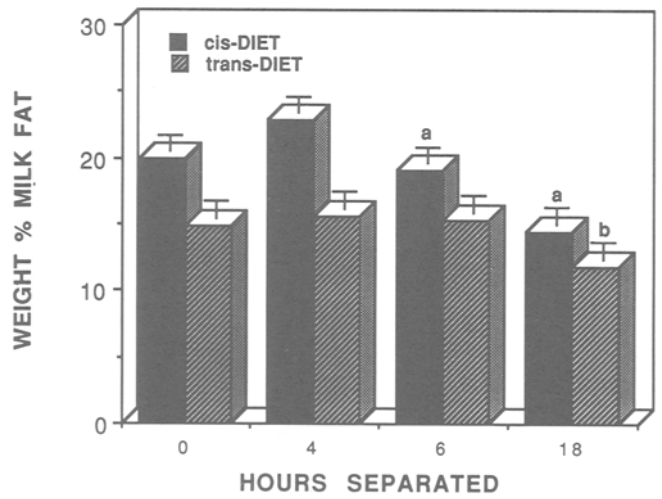

FIG. 4. Percentage of milk fat from C57Bl/6J mice separated from their pups prior to milking. Means and SEM are shown. See Figure 3 for sample sizes. The "a" and "b" signify values different from 0 h of that diet (*t*-test $P \leq 0.03$). Except for the 18 h values the *trans* Diet values are significantly less than *cis*-Diet values ($P < 0.01$).

Fatty acid composition of murine milk fat. Fatty acid compositions of milk fat were monitored throughout the lactation and preconditioning studies. A summary of data for milks obtained at 10-d lactation is given in Table

TABLE 3

Fatty Acid Composition of Milks at Day 10 of Lactation[a]

Major fatty acids[c]	Fatty acid methyl ester wt%[b] C-Diet[d]	T-Diet[d]
≤16:0	21 ± 3	23 ± 3
16:0	24 ± 3	19 ± 1
18:0	2 ± 0.2	2 ± 0.1
16:1*c*	4 ± 0.7	4 ± 0.8
18:1*t*	ND	8 ± 0.6
18:1*c*	40 ± 5	28 ± 8
18:2n-6	8 ± 1.5	11 ± 2.0
18:2*i*	ND	0.7 ± .2
20:4	0.8 ± 0.3	1.4 ± 0.6
Summary of 18 carbon fatty acids		
18:0	2	2
18:1*c*+*t*	40	36
18:2n-6+*i*	8	12
Total 18 carbon	50	50

[a]Pups remained with dams until milking.
[b]Means ± SD. n, 12 C-Diet, 5 T-Diet.
[c]Fatty acid carbon number:double bonds; *c*, *cis* isomers; *t*, *trans* isomers; *i*, isomers, both *cis* and *trans* of 18:2n-6.
[d]Diets are defined in Tables 1 and 2. Diets differ in the composition of 18:1 and 18:2 isomers. In the T-Diet about half of the 18:1 is *trans* geometrical isomers. ND, not detected.

3. Fatty acid composition within a diet group was similar throughout the lactation study. The milk fatty acid composition reflected the dams' diet in that milk from both diet groups contained 18:2n-6 and the T-Diet samples contained *cis* and *trans* isomers derived from the partially hydrogenated fat in the diet.

Data for groups of fatty acids in preconditioned milks are given in Figure 5 as percent of the total FAME. There were no differences in fatty acid compositions within a diet group until 18 h of preconditioning, when the *de novo* group of fatty acids (<16 carbons) were dramatically decreased and the proportion of 18 carbon acids, derived from diet and adipose stores (17), were increased. However, when the decrease in total fat also was taken into account, the *de novo* group was severely decreased and the other fatty acid groups were slightly decreased or present at nearly the same level as was found at the 0-h point (data not shown).

These alterations in fatty acid composition coupled to the lack of an increase in total fat suggest that dramatic changes have occurred in the mammary gland at 18 h of preconditioning. The changes in fatty acid composition are consistent with those observed by Hurley *et al.* (18) in dairy cows during early involution and are similar to those of Grigor *et al.* (19), who reported decreased percentages of 12:0 and 14:0 and increased 18:0 in milk from glands of rat dams suckling two pups as compared to those suckling 10 pups. The dramatic alterations in milk fat after the 18-h preconditioning period in the present studies are probably due to physiological changes associated with mammary gland involution.

In summary, the studies suggest that extensive preconditioning prior to milking can yield inaccurate and potentially misleading data. Since there was no significant increase in the volume of milk obtained when dams were preconditioned beyond 4 h, we recommend that the preconditioning period, if employed, not exceed 4 h.

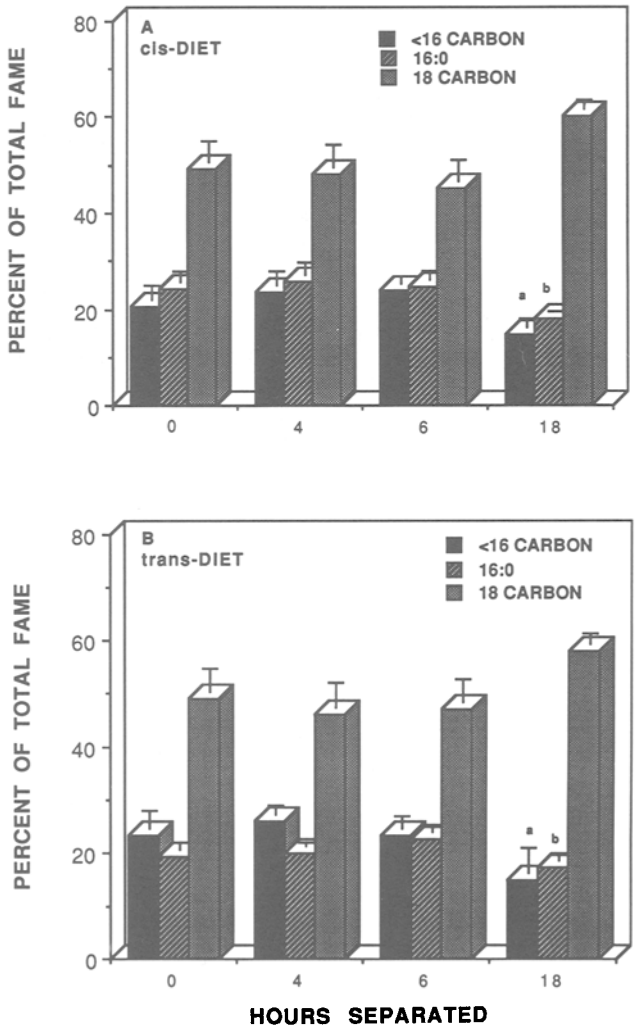

FIG. 5. Selected fatty acids in milk of C57Bl/6J mice after pups are separated from dams. Means and SEM are shown for the *cis*-Diet groups (A) and the *trans* Diet groups (B). Sample sizes for 0, 4, 6 and 18 h are 12, 5, 5 and 3 for *cis* Diet and 5, 5, 6 and 3 for *trans* Diet, respectively. The "a" and "b" indicate significantly lower values ($P \leq 0.05$) than at earlier times.

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Bile Salt Stimulated Lipase: Comparative Studies in Ferret Milk and Lactating Mammary Gland[1]

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Bile salt stimulated lipase (BSSL) activity is 10–20 times higher in ferret milk than in human milk. We have used the ferret to study BSSL activity in lactating mammary gland and in mammary cells isolated by hyaluronidase-collagenase treatment followed by Ficoll gradient centrifugation. Furthermore, we have compared the characteristics of BSSL in the tissue preparations (homogenate or cells) to BSSL of ferret milk and to BSSL purified from ferret and human milk. The characteristics of BSSL in ferret mammary gland preparations and milk were similar to those of human milk BSSL—absolute requirement of primary bile salts, pH optimum of 7.5–9.0, stability at pH 3–9 and inhibition by eserine (physostigmine) and by serum. Purified ferret milk BSSL had a lower molecular weight (90kD) than did human milk BSSL (125 kD). There was an 86% homology of the *N*-terminal amino acid sequence between BSSL of ferret and of human milk. The marked similarity in characteristics between BSSL in ferret and human milk and the high activity of BSSL in ferret milk (520 U/mL colostrum and 250 U/mL mature milk) indicate that this species is an ideal animal model for the study of the synthesis and secretion of this digestive lipase which constitutes a significant portion (1–2%) of total milk protein.
Lipids 27, 917–922 (1992).

The presence and functional significance of bioactive components in human milk have been elucidated (1–3) only recently. Although enzyme activities of human milk have been demonstrated by *in vitro* studies (1), *in vivo* studies of the mechanisms of synthesis and secretion of these milk components have not been possible.

Bile salt stimulated lipase (BSSL), an enzyme previously found in the milk of humans (4,5), high primates (6), and carnivores such as the dog and cat (7), represents a significant proportion of total milk protein (1–2%) (8,9) and is probably the most abundant of the enzyme components in milk. BSSL is functionally similar and immunologically homologous to pancreatic carboxyl esterase (10). Because pancreatic lipase activity is low in the infant during the first months of life (11), it has been suggested that BSSL serves a compensatory role in fat digestion in the infant (5,12–15). Although several studies have assessed the possible compensatory role of this enzyme in the digestion of fat in the newborn (12–15), only one study has addressed localization of the enzyme in the lactating mammary gland (16).

In the present study, we have used a new animal model (the ferret) to quantitate and to compare the characteristics of the activity of BSSL in milk, in mammary gland and in cells isolated from the lactating mammary gland to those of the enzyme purified from ferret milk, as well as to known characteristics of BSSL activity of human milk.

MATERIALS AND METHODS

Two primiparous ferret jills, species *Mustela putoris furo* (Marshall Farms, North Rose, NY) were obtained in late pregnancy. The animals were housed separately in 18 × 10-inch stainless steel cages containing a four-inch bed of cedar shavings (17). Both jills received Purina Ferret Chow (Ralston Purina, St. Louis, MO) and water *ad libitum*. Kits were born at term on day 43 of gestation.

Milk collection. Milk was collected from each jill on days 3 (colostrum), 7, 10 and 15 of lactation. The jills were lightly sedated with acepromazine (TechAmerica Group Inc., Elwood, KS) and 2–5 units of oxytocin (pitocin, Parke Davis Division of Warner Lambert Co., Morris Plains, NJ) were injected intramuscularly to initiate milk let-down. Milk was manually expressed from each teat, collected by capillary action into a pasteur pipette and immediately transferred to a collection tube held on ice. Milk specimens were frozen at −70°C within 45 min from initiation of collection.

Mammary biopsy/tissue collection. Samples of mammary tissue were taken from each jill on day 10 (by sterile surgery) and on day 14 (sacrifice). Three hours prior to surgery, kits were separated from jills and placed in an incubator (70% humidity; 32°C). Milk was fully expressed from each teat prior to surgery. Ferrets were sedated with telezol (Fort Dodge Labs, Fort Dodge, IA) (14 mg/kg) delivered intramuscularly. The entire lobe of the left anterior mammary gland was removed through a half-moon incision and immediately placed on ice. Subsamples of the gland were rinsed in saline and frozen at −70°C, or used for cell dissociation. On day 14 *postpartum*, jills were sacrificed by an overdose of pentobarbital and exsanguination by cardiac puncture. Mammary tissue in the right inguinal area was perfused through the right femoral artery with 30 mL cold saline. The gland was removed and subsampled for frozen storage at −70°C. The left inguinal and right anterior glands were then removed, rinsed in saline and either frozen at −70°C or used for cell dissociation.

Mammary cell isolation. A mammary cell suspension enriched in secretory epithelial cells was prepared as previously described (18). Briefly, 0.25 to 10 g of mammary tissue were minced and subjected to enzymatic digestion with hyaluronidase and collagenase (100 U/mL and 150 U/mL, respectively; Worthington, Freehold, NJ) during 25–30 min incubation at 37°C with constant infusion of O_2/CO_2 (95:5%). The isolated cells were counted under light microscopy using a Neubauer homocytometer and

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Abbreviations: BSSL, bile salt stimulated lipase; HPLC, high-performance liquid chromatography; SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis.

crystal violet staining. When approximately 1×10^6 cells per mL of suspension were present, the digestion process was stopped by addition of trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) and slow cooling on ice. The suspension was filtered and subjected to a series of three washings and sedimentations on a 30% discontinuous Ficoll gradient (18). The cell band was collected and diluted to approximately 1×10^6 cells/mL before freezing. DNA from both tissues and cells was extracted by the method of Schneider (19) and quantitated by the method of Burton (20). Protein content of milk and purified enzyme was determined by the method of Lowry *et al.* (21).

Purification of BSSL. BSSL was purifed from ferret milk using a small-scale version of the method developed by Blackberg and Hernell (18). Molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on both 11–23% gradient (22) and 7.5% total acrylamide concentrations according to the methods of Laemmli (23). *N*-Terminal amino acid sequence was determined using an Applied Biosystems (Foster City, CA) Gas Phase Protein Sequencer (Model 470A) and a Waters Associates (Milford, MA) HPLC (high-performance liquid chromatography) System.

Assay of BSSL. Activity of BSSL was measured using glycerol tri[9,10-(n)^{3}H]oleate as substrate (24). The triglyceride was emulsified at 4°C with a Branson Model 200 Sonifier (Branson Sonic Power, Danbury, CT) for two 2-min intervals at setting 3, 50% of time pulsed. The standard assay system contained 1.6 mM triolein, 60 mM Tris-HCl buffer pH 8.5, 150 mM NaCl, 12 mM sodium taurocholate, 2.8% bovine serum albumin and dilute enzyme in a total volume of 200 μL. After incubation at 37°C for 15 min, the reaction was stopped by the addition of a mixture of chloroform/methanol/heptane (1.25:1.41:1, vol/vol/vol). The [^{3}H]oleic acid released was separated by liquid-liquid partition (25) and quantitated by liquid scintillation spectrometry with automatic external standardization as previously described (7). One unit of lipase activity is the release of 1 μmol of fatty acid per min. A milk sample of known BSSL activity was included in each assay to verify accuracy and reproducibility of the assay.

RESULTS

Concentration of BSSL in ferret milk during lactation. The activity of BSSL in ferret colostrum and milk collected on days 3, 7, 10 and 15 of lactation is shown in Figure 1. Additional samples of ferret milk (Marshall Farms) at various points throughout lactation were tested for comparison and are included in Figure 1. Marked differences were evident between the activity of BSSL in colostrum produced by each jill (310–634 units mL). However, beyond day 7 of lactation, activity of BSSL remained relatively constant in a range of 250 units/mL among different jills.

Purification of BSSL. Data for the purification of BSSL from ferret milk are presented in Table 1. There were differences in the partition of BSSL into whey and casein fractions between ferret milk and human milk. Initial purification of BSSL from human milk in this laboratory has resulted in 80–90% of total BSSL activity remaining in the whey fraction. Furthermore, loss of enzyme protein was appreciable only after acidification and incubation at 40°C for removal of casein. However, only 51% of the

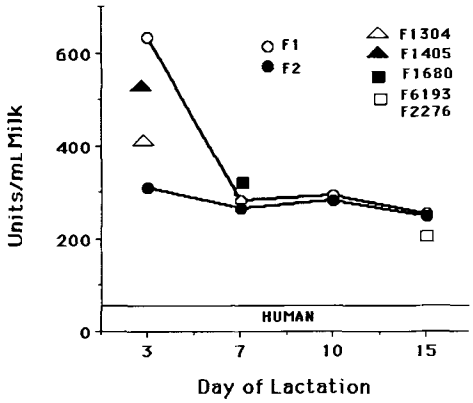

FIG. 1. Bile salt stimulated lipase (BSSL) activity in ferret colostrum and milk. Longitudinal data are presented for two ferrets. Additional data are provided for individual specimens of ferret colostrum and milk. Average activity of BSSL in human milk during the first two weeks of lactation is given for comparison.

BSSL in ferret milk was associated with the whey fraction. Centrifugation (10,000 × *g* for 1 h) alone resulted in significant precipitation of BSSL protein (17%) from ferret milk, whereas acidification and incubation at 40°C resulted in additional loss of enzyme in the precipitating fraction. The remaining (16%) BSSL activity not accounted for is most likely associated with the lipid fraction as this amount was lost after the defatting stage.

BSSL purified in this laboratory (26), as well as by others (8,9), has been shown to represent 1–2% of total milk protein. Data from the purification of BSSL in ferret milk indicate that the enzyme represents 8% and 3% of total protein in colostrum and in milk collected at day 18 of lactation, respectively. The molecular weight of BSSL in ferret milk as determined by SDS-PAGE is 90,000 Daltons. The molecular weight of the BSSL in ferret milk is identical to the molecular weight of BSSL in the milk of the cat (27), a close relative of the ferret, but is somewhat smaller than the human form [determined in this and other laboratories to be 125,000 Daltons (8,28,29) (Fig. 2)].

The *N*-terminal amino acid sequence determined for BSSL purified from ferret milk is shown in Table 2. The amino acid sequence of BSSL in the milk of the ferret shows 86% homology to the known amino sequence of human BSSL (9). There were differences between ferret and human BSSL at amino acids 2, 11 and 18 (Ser, Gly and His, respectively). In all cases these amino acids substituted for lysine residues in the BSSL of human milk.

Enzyme kinetics. Kinetics of BSSL purified from ferret milk and those of BSSL in whole ferret milk are presented in Figure 3. The substrate concentration curves for BSSL in ferret and in human milk are shown in Figure 4. While BSSL purified from ferret milk showed a hyperbolic response to increasing substrate concentration, the response of human milk BSSL was sigmoidal with a lag phase at substrate concentrations below 0.8 mM and inhibition at substrate concentrations greater than 3.2 mM. The apparent Km of BSSL in ferret milk was 1.67 mM. The apparent Km of human milk BSSL was close to that of the ferret enzyme, ranging between 1.6–1.7 mM.

Characterization of BSSL in ferret milk, in mammary tissue and in mammary cells and comparison to BSSL

TABLE 1

Purification of Ferret Milk BSSL[a], Comparison with the Purification of BSSL in Human Milk

Purification step	Milk fraction	Ferret Volume (mL)	Ferret Total activity (units; U)	Ferret U/mg protein	Human Volume (mL)	Human Total activity (units; U)	Human U/mg protein
Whole milk		5.6	1256	3.53	58	1393	1.1
Defatted	Skim	3.5	841	4.26			
	precipitate		212				
Acidified	Whey	2.7	650	6.64	83	139	2.2
(40°C)	casein	2.7	180	2.80			
Heparin Sepharose I		15.3	293	15.75	26.5	609	15.6
Heparin Sepharose II		11.1	194	108.80	9.7	54	66.6

[a]BSSL, bile salt stimulated lipase.

TABLE 2

***N*-Terminal Amino Acid Sequence of Bile Salt Stimulated Lipase (BSSL) Purified from Human or Ferret Milk**

Human[a]	Ala	Lys	Leu	Gly	Ala	Val	Tyr	Thr	Glu	Gly	Lys	Phe	Val	Asn	Lys	Lys	Leu	Gly	Leu			
		2									11				15							22
Ferret	Ala	Ser	Leu	Gly	Ala	Val	Tyr	Thr	Glu	Gly	Gly	Phe	Val	Asn	His	Lys	Leu	Gly	Leu	Phe	Gly	Asp

[a]Data on human milk from ref. 9.

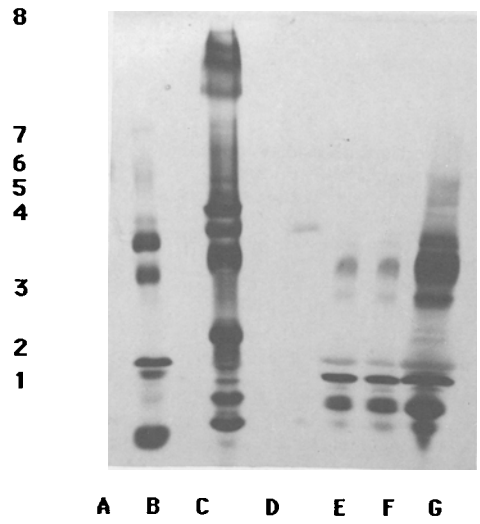

FIG. 2. Sodium dodecyl sulfate polyacylamide gel electrophoresis of purified ferret and human milk bile salt stimulated lipase (BSSL). Lanes: A, BSSL purified from ferret milk; B, BSSL purified from human milk; C, human milk, skim milk fraction; D, standards for molecular weight; E, BSSL purifed from ferret milk; F, ferret milk casein pellet, second centrifugation; G, ferret milk casein pellet, first centrifugation; H, ferret milk, skim milk fraction. Standards for molecular weight (kD), lane D: 1, lysozyme (14.4 kD); 2, trypsin inhibitor (22 kD); 3, carbonic anhydrase (31 kD); 4, ovalbumin (45 kD); 5, serum albumin (66 kD); 6, phosphorylase B (97 kD); 7, β-galactosidase (116 kD); 8, myosin (200 kD).

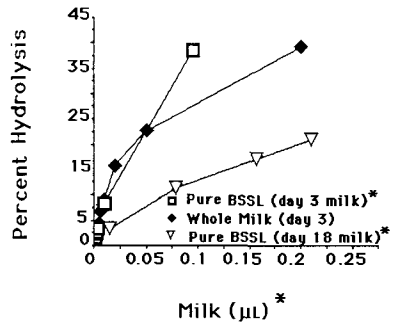

FIG. 3. Bile salt stimulated lipase (BSSL) enzyme concentration curve. Comparison of activity of pure ferret milk BSSL (expressed in equivalent milk volume based on purification data, Table 1) with BSSL activity in whole milk.

purified from ferret milk. The effect of bile salts on the activity of BSSL purified from ferret milk, in whole ferret milk, mammary tissue and mammary cells (0–30 mM taurocholate or glycocholate) is shown in Figure 5. Maximal enzyme activity for whole milk and for purified BSSL was achieved at taurocholate concentrations of 12–15 mM, whereas BSSL in mammary tissue and mammary cells was maximally activated at 10 mM taurocholate. In terms of absolute values, at 10 mM taurocholate, BSSL activity in the mammary tissue was 6.2-fold greater than cellular BSSL. Inhibition of BSSL activity in mammary tissue and mammary cells occurred at concentrations greater than 12 mM taurocholate, with 51–57.5% inhibition of maximum activity at 30 mM taurocholate. Likewise, purified BSSL and BSSL in whole milk showed inhibition in activity at taurocholate concentrations greater than 20 mM; however, at 30 mM taurocholate inhibition was somewhat less (34.2 and 46%, respectively) than that of the enzyme in mammary tissue and mammary cells.

BSSL activity in whole milk, in purified enzyme, in mammary tissue and in mammary cells was maximal in the presence of 10 mM glycocholate but declined in activity at glycocholate concentrations greater than 10 mM.

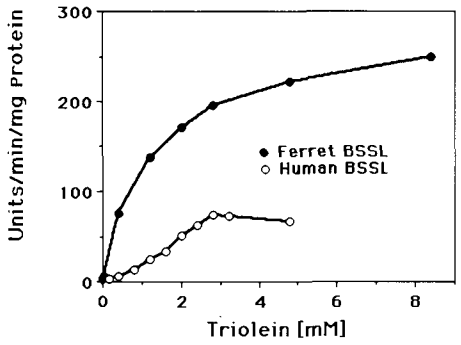

FIG. 4. Substrate concentration curve for bile salt stimulated lipase in ferret milk and in human milk.

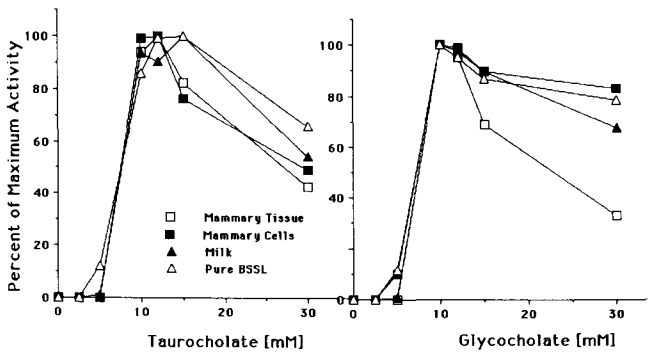

FIG. 5. Effect of bile salts, glycocholate and taurocholate, on bile salt stimulated lipase activity of ferret mammary tissue, mammary cells, milk and the purified enzyme. No enzyme activity was detected in the presence of secondary bile salts.

BSSL activity in mammary tissue was most affected with 67% inhibition at 30 mM glycocholate as compared to 17-32% inhibition of purified, whole milk and cellular BSSL. The response of BSSL was greater to glycocholate than to taurocholate at all bile salt concentrations: enzyme activity in cells, tissue and whole milk was 120-170% greater in the presence of glycocholate as compared to taurocholate; purified BSSL was 300-415% more active with glycocholate than with taurocholate. Secondary bile salts did not elicit BSSL activity in milk, mammary tissue or cells. In human milk, BSSL activity was slightly higher with glycocholate than taurocholate in the range of 10-15 mM, and no activity was detected in the presence of secondary bile salts.

Effect of pH. The effect of pH on the activity of BSSL in milk, purified enzyme, mammary tissue and mammary cells is shown in Figure 6. Maximal activity of purified BSSL and BSSL in whole milk was achieved at pH 8.0, and mammary tissue and mammary cells reached optimum activity at pH 8.5. At pH 7.5-9.0, greater than 75% of BSSL was present in whole milk, purified enzyme and mammary cells. No activity was detected in mammary tissue homogenate below pH 8.0 or above pH 9.0.

Stability of BSSL as function of pH. Dilute samples of purified BSSL, ferret milk, mammary tissue and mammary cells were incubated at 37°C for up to 60 min at pH 1.5-9.5. Samples were assayed after 0, 30 and 60 min of pre-incubation at optimal conditions (pH 8.5, 12 mM taurocholate) to determine the stability of enzyme activity. Stability of BSSL to 60 min pre-incubation is shown in Figure 7. Greater than 75% of BSSL was present in whole milk after 60 min of preincubation at pH 4.0-9.0. BSSL purified from ferret milk retained 75% of control activity when pre-incubated for 60 min at pH 8.5, but only 25-30% of initial activity was measurable after pre-incubation at pH above or below optimum. In comparison to purified BSSL and whole milk BSSL, the BSSL activity in mammary tissues and mammary cells was more stable at extremes of pH. As compared to initial activity, mammary cells showed enhanced activity after 60-min pre-incubation at pH 3.0.

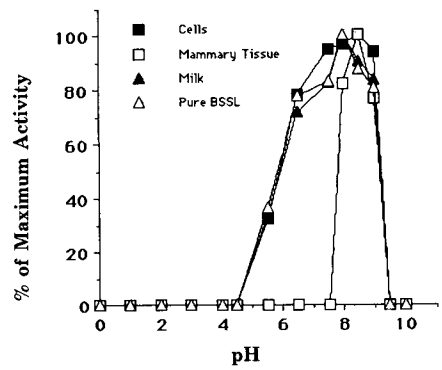

FIG. 6. The effect of pH on bile salt stimulated lipase (BSSL) activity. Comparison of the activity of purified ferret milk enzyme with that of BSSL in ferret mammary tissue, mammary cells and ferret milk. The standard assay system was adjusted to the various pH values using buffers at a final assay concentration of 60 mM. Buffers used were: pH 2.4-3.6 glycine-HCl; pH 3.6-6.0 Na acetate; pH 6.0-7.5 Na phosphate; pH 7.5-8.5 Tris-HCl; and pH 8.5-9.5 glycine-NaOH.

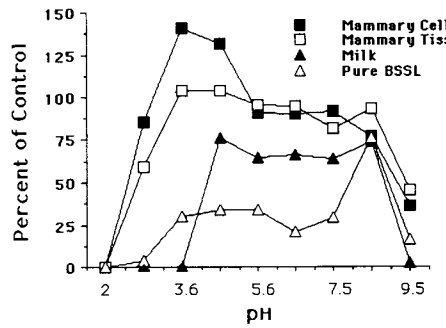

FIG. 7. Stability of bile salt stimulated lipase (BSSL) as a function of pH. Mammary tissue, mammary cells, milk and purified enzyme were incubated in 0.05 M buffer (see legend to Fig. 6) for 60 min at a pH range of 2.0-9.5, followed by incubation in the standard assay system (pH 8.5) for quantitation of BSSL activity. Note: the X axis is not drawn to scale.

Inhibition of BSSL activity by eserine hemisulfate and serum. Both eserine (physostigmine) and serum are potent inhibitors of milk BSSL (24). Activity of BSSL in milk, mammary tissue and mammary cells was measured in the presence of 0.1 to 10 mM eserine hemisulfate (Fig. 8). In all cases, less than 10% of maximum activity remained when 2 mM eserine was included in the assay.

Activity of non-perfused mammary tissue homogenate was compared to activities of purified BSSL, ferret milk and serum-free mammary cells in the presence of 0.5-5% heat-inactivated serum (vol/vol) (Fig. 9). At concentrations

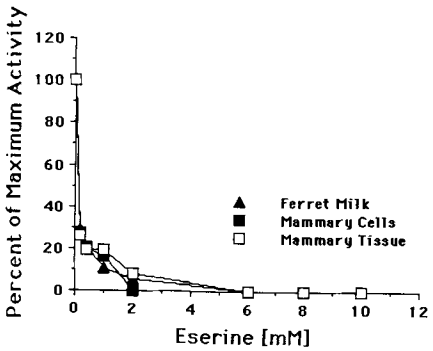

FIG. 8. Effect of eserine (physostigmine) on bile salt stimulated lipase activity in ferret milk, mammary cells and mammary tissue.

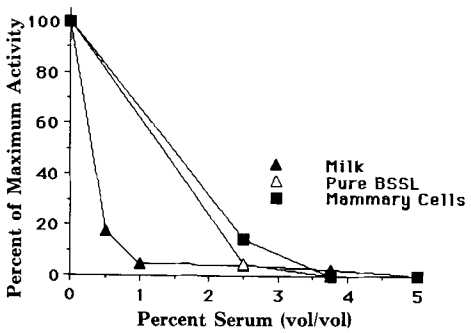

FIG. 9. Effect of serum on bile salt stimulated lipase activity in ferret milk, mammary cells and mammary tissue.

of 0.5% serum, less than 20% of maximum activity was measured in whole milk, and complete inhibition was reached with 4% serum. The nature of the BSSL inhibitor in serum is not known. Recent studies have established that the serum inhibition of acid and neutral cholesteryl ester hydrolases is due to serum apoprotein A-I (30). Whether milk BSSL, an enzyme identical to pancreatic carboxylester hydrolase (10,31), is likewise inhibited by apoprotein A-I remains to be established. It is of interest that while BSSL activity is inhibited by serum, a lipolytic activity stimulated by taurocholate was measured in the serum of the ferret (275 nmol/min/mL serum). This serum lipase did not contribute to mammary homogenate lipolytic activity at the concentration of tissue assayed (0.01 mg).

DISCUSSION

This study describes the strong similarities in both structural and functional characteristics of BSSL produced by the ferret and by the human. Ferret milk contains 10–20 times higher BSSL activity than human milk. This difference does not appear to be a result of higher quantities of BSSL protein in mature ferret milk (after day 7 of lactation) but is more likely due to the greater Vmax (160% of human) and higher turnover number (5800 sec^{-1}) of BSSL in ferret milk as compared to human BSSL. The data demonstrate the presence of BSSL in the mammary gland and in the mammary cells of the lactating ferret. Although this study does not delineate the site of synthesis for BSSL, the data suggest that the enzyme is synthesized in the mammary gland. Although a bile-salt activated lipase is present in the serum of the lactating ferret (probably pancreatic lipase or carboxylester hydrolase), it is unlikely that this is the source of BSSL in milk as there is a greater than 1000-fold difference in milk BSSL activity as compared to serum bile salt stimulated lipolytic activity. Comparison of the activity of BSSL in mammary tissue homogenate to activity of cellular BSSL shows a 3–5-fold greater activity of BSSL in tissue homogenate (expressed per μg DNA). This observation may indicate that synthesis of BSSL is specific to a particular cell type or that enzyme activity might be lost during the cell isolation procedure. A cell preparation enriched in epithelial cells was used in this study. However, the BSSL in mammary cells also may be in a bound form either at the cellular or subcellular membrane or may somehow be modified once transported from the cell. The time dependent increase of BSSL activity in mammary cells at low pH may represent the lysis of Golgi vesicles transporting protein from the cell or release of BSSL from a membrane-bound receptor.

The physiological mechanisms of lactation are poorly understood, particularly with respect to specialized bioactive components in milk. BSSL is a particularly unique component of milk since it possesses no known function in milk nor in the lactating mammary gland. Comparison of species with and without BSSL shows a strong evolutionary link in species secreting BSSL. Milk of ruminants, rodents and omnivorous mammals has no BSSL activity (32,33), whereas BSSL is secreted in milk produced by carnivores (7,33). Further study of this protein is necessary to elucidate the reasons for species differences. Human milk also contains large quantities of amylase (34,35) which, likewise, has no known function in milk or in the lactating mammary gland. Study of genetic similarities in mammary and pancreatic cells may provide evidence of an evolutionary expression of DNA coding for these enzymes as the mammalian species evolved.

While the evolution of lactation and the origin of bioactive components in human milk are still unknown, the functional significance of BSSL to the neonate cannot be ignored. Once ingested by the infant, BSSL is active in the digestion of milk lipid in the intestine. Because pancreatic function of the human infant is not fully developed in early life, this bioactive component is important for the neonate, especially the premature (36). Wang *et al.* (27) reported a greater rate of growth in kitten fed milk replacer supplemented with purified human BSSL. Similarly, addition of human milk to low-birth-weight infant formulas resulted in a lower excretion of fat by very premature infants as compared to infants fed formula alone (37). Furthermore, fat absorption is lower in preterm infants fed pasteurized as compared to fresh human milk (38).

Use of the ferret as an animal model will allow detailed investigation of the mechanisms of BSSL synthesis in the mammary gland and secretion into milk throughout lactation. Furthermore, animal studies will enable the investigation of the regulation of *prepartum* synthesis and secretion of BSSL. Preliminary studies indicate that BSSL is present in human *prepartum* mammary secretions as early as three months before full term delivery (39). In addition, the use of the ferret will allow study of the *in vivo* role of BSSL in fat digestion in the newborn's

intestine and its impact on normal growth and development.

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Neutral Glycolipids of Human and Bovine Milk[1]

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The neutral glycolipids of milk, a small fraction of the total lipids, are of potential biological importance. The simultaneous quantitation of the simple (less than five sugars) glycosphingolipids of human milk samples was achieved by high-pressure liquid chromatography. The samples, representing various stages of lactation, parity of the nursing child, and age of the mother, contained similar glycolipid patterns, but with varying individual glycolipid concentrations. The cerebrosides are major glycosphingolipids of human milk: the non-hydroxylated fatty acid (NFA)-containing species are present at 1.8 μM, and the hydroxylated and/or short-chain fatty acid-containing species (HFA) are present at 1.7 μM; NFA lactosylceramide is present at 931 nM. The cerebrosides appear to be primarily galactosylceramides (galactocerebrosides); glucosylceramides (glucocerebrosides) are a minor component. Globotriaosylceramide (Gb_3) is found at 50 nM and 73 nM for the NFA and HFA species, respectively, while globoside (Gb_4) is found at 45 nM and 46 nM for the NFA and HFA species. Bovine milk glycosphingolipids differ from those of human milk, with bovine milk containing mainly NFA glucosylceramide (8 μM) and NFA lactosylceramide (17 μM); bovine milk contains little Gb_3 or Gb_4.
Lipids 27, 923–927 (1992).

Many reports now indicate that breast-fed infants may have a lower incidence of morbidity and mortality than artificially-fed infants, not only in less developed nations with high endemic levels of pediatric disease (1), but also in more developed countries (2,3). Although many factors could contribute to this phenomenon, non-immunoglobulin protective factors found in human milk are receiving increasing recognition as contributors to human protection. For example, the milk ganglioside GM_1 binds to cholera toxin and labile toxin of *Escherichia coli* (4), and to a related toxin produced by *Campylobacter jejuni* (5).

Many other glycolipids are able to bind to pathogens. The simple (less than five sugars) neutral glycolipids of the globo-series which are a common feature of most mammalian membranes are shown in Figure 1. Gb_3 (globotriaosylceramide), with its terminal galactose α1-4 galactose, is known to bind to Shiga toxin I (6) and to Shiga-like toxin types 1 and 2 (7,8), as well as to the P pili of uropathogenic *E. coli* (9). Gb_4 (globoside), whose galactose α1-4 galactose moiety is found in the internal sequences of the molecule, binds to P pili (10) and to the type 2 variant of Shiga-like toxin (11). Thus, the presence of such lipids in milk would be consistent with the general hypothesis that human milk contains non-immunoglobulin compounds capable of competing with infantile host cell receptors for binding to pathogens, thereby conferring protection to the nursing infant.

Glycolipids have been investigated in ruminant and human milk: over half of the glycolipids of caprine milk and 70% of the glycolipids of bovine milk are associated with the milk fat globule membrane (MFGM). Bovine MFGM contains glucocerebroside and lactosylceramide as its major constituents; both of these glycolipids contain mainly non-hydroxy fatty acids (NFA), and lactosylceramide is present at slightly higher concentrations than cerebroside (12). Human MFGM also contains cerebroside and lactosylceramide as its major constituents. However, it contains four times the total glycolipids as bovine MFGM, galactocerebroside accounts for 88% of the total monohexosylceramide, 20% of the fatty acid residues are hydroxylated fatty acids (HFA), and lactosylceramide is present in much lower concentrations than the cerebrosides (13) (see structures in Fig. 1). The molecular species of cerebrosides found in the bovine MFGM differed from those found in the skim milk; MFGM cerebrosides contained primarily long-chain fatty acids, while those in the skim milk contained fatty acids primarily of 18 carbons and shorter.

To our knowledge, the degree of individual variation in milk glycolipid levels has not been determined. This is of particular interest because of the potential for these milk

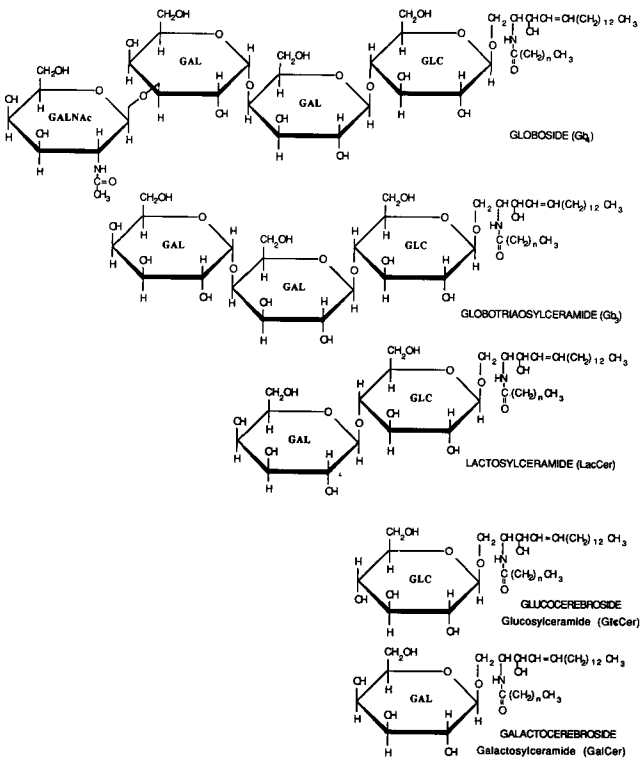

FIG. 1. Structures of the neutral glycosphingolipids measured in human milk.

[1]Based on a paper presented at the Symposium on Milk Lipids held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

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Abbreviations: Gb_3, globotriaosylceramide; Gb_4, globoside; HPLC, high-pressure liquid chromatography; HFA, hydroxylated fatty acid; NFA, non-hydroxylated (normal) fatty acids; MFGM, milk fat globule membrane.

glycolipids to alter binding of enteric pathogens in the infant.

The present study had two purposes: The first was to compare the glycolipid profiles of whole human and whole bovine milk, with particular interest in Gb_3 and Gb_4 levels. The second was to measure the degree to which the concentrations of these glycolipids vary among human milk samples obtained from a diverse population of healthy lactating mothers and how they vary among commercially available bovine milk samples.

EXPERIMENTAL PROCEDURES

Milk samples. For qualitative analysis of bovine milk, a cow in the Boston area was milked, and, within 30 s, aliquotted directly into methanol to produce a sample that was subjected to no further storage or processing prior to glycolipid analysis. For quantitative analysis, five samples of pooled bovine milk from different processing plants were obtained commercially, and duplicate aliquots of each sample were analyzed on the day of purchase. Human milk samples were obtained from the Central Massachusetts Regional Milk Bank, Worcester. Thirty random samples were pooled for a representative human milk glycolipid profile to be compared with the bovine profile. Sixteen individual samples were selected to represent a wide range of maternal age (mean, 29 yr; SD, 5 yr; range, 20–37 yr), ethnicity, parity of the child (primiparous, 5; secondiparous, 7; tertiparous, 2; quadriparous, 2), and stage of lactation (1–17 mon).

Duplicate aliquots of each sample were utilized to quantitate the concentrations of the individual glycolipids in human milk, and to determine the degree to which they vary among individuals. Human samples were frozen immediately after pumping, stored for a period of up to ten days at −20°C, and transported and stored at −80°C until analysis.

Glycolipid extraction. Duplicate aliquots of 3 mL of well mixed milk samples were placed into 15-mL screw-capped glass culture tubes. Methanol (4 mL) and chloroform (8 mL) were added sequentially with vigorous mixing to establish the Folch lipid distribution (14). After centrifugation, the upper phase was removed, and the lower phase was rinsed with 2 mL of chloroform/methanol/water (3:48:47, vol/vol/vol; theoretical upper phase), filtered through glass wool, and dried and the residue was dissolved in 200 μL of chloroform and applied to a 125-mg silicic acid column (15). The column was washed with 3 mL of chloroform, and the lower phase glycolipid fraction was eluted from the column with 4 mL of acetone/methanol (9:1, vol/vol). The dried glycolipid fraction was subjected to mild alkaline hydrolysis for one hour at ambient temperature with 1 mL 0.6M NaOH in methanol and 1 mL of chloroform to hydrolyze glycerol-derived fatty acid esters. After 1 h, the reaction was stopped with 1.5 mL 0.4M HCl_{aq} and Folch distribution established with the addition of 1 mL methanol and 3 mL chloroform; the upper phase, containing salts and hydrolysis products, was discarded; the lower phase was washed twice with theoretical upper phase, and the glycolipid fraction was dried and lyophilized.

Perbenzoylation. A fresh solution of 10% benzoyl chloride in dry pyridine (0.5 mL) was added to each glycolipid sample; after 16 h at 37°C, the sample was dried under nitrogen (16). Hexane (3 mL) and 80% $methanol_{aq}$ saturated with Na_2CO_3 (2 mL) were added, mixed vigorously for 1 to 2 min, and the lower phase was discarded. The upper phase (hexane) was rinsed five times by vigorous mixing with fresh lower phase, three times with 80% methanol, and then dried under nitrogen to yield the dry perbenzoylated glycolipids.

High-pressure liquid chromatography (HPLC). Perbenzoylated glycolipids were dissolved in CCl_4 and separated on a 50-cm × 2.1-mm column of Zipax uncoated silica (Rockland Technologies, Newport, DE; obtained through Mac-Mod Analytical, Chadds Ford, PA), using a 16-min linear gradient from 1% to 20% dioxane in hexane at 2 mL/min (17). Absorption at 229 nm was monitored on a Macintosh SE computer running Dynamax software (Rainin, Woburn, MA). Peaks were identified by co-elution with authentic commercial standards (Matreya, Pleasant Gap, PA), as well as co-elution with our own standards whose identities were previously determined by chemical and mass spectrometric methods. The identity of Gb_3 was further confirmed by its binding to pure Shiga toxin I (gift of Thomas G. Cleary, Houston, TX). Quantitation was derived from the area under the peaks. Glycolipid recovery from the original samples was 70 ± 10% by this method, and values given have been corrected for losses.

Statistics. The square of Pearson's product-moment correlation coefficient r^2 was calculated (18) for each glycolipid across duplicate analyses of milk samples from 16 different mothers to determine the proportion of the variance common to both sets of duplicate data, which we used to differentiate the biological variation (variance in common) from that inherent to our analytical method (random variance).

RESULTS

The glycolipid pattern of the unprocessed milk sample obtained from an individual cow was similar to that from commercially obtained bovine milk, which is pooled and processed; however, the individual sample contained low but measurable levels of Gb_3, while Gb_3 was rarely detected in any commercial bovine milk samples in our laboratory. The individual bovine sample was used in Figure 2 to compare the neutral glycosphingolipid HPLC profile of an individual human milk sample (upper panel) with that of an equivalent bovine milk sample (lower panel). The major constituents of whole bovine milk are glucocerebroside and lactosylceramide (see Fig. 1 for structures) of the non-hydroxylated fatty acid-containing species. In contrast, the principal constituents of whole human milk are the galactocerebrosides containing both NFA and HFA, and, to a lesser extent, NFA lactosylceramide. The shoulder on the leading edge of the NFA galactocerebroside peak was found to be NFA glucocerebroside by a separate analysis. Small but appreciable quantities of NFA and HFA Gb_3 and NFA and HFA Gb_4 are found in human milk; whereas this bovine milk sample contains less Gb_3 and no Gb_4. The compilation of five independent analyses of pooled bovine milk is shown in Table 1.

It is striking that the dominant glycolipid of bovine milk is NFA lactosylceramide, which, at a concentration of 17 μM, represents 64% of the neutral glycosphingolipid fraction. NFA glucocerebroside, at 8 μM, represents another 29% of this fraction, and HFA cerebrosides, at

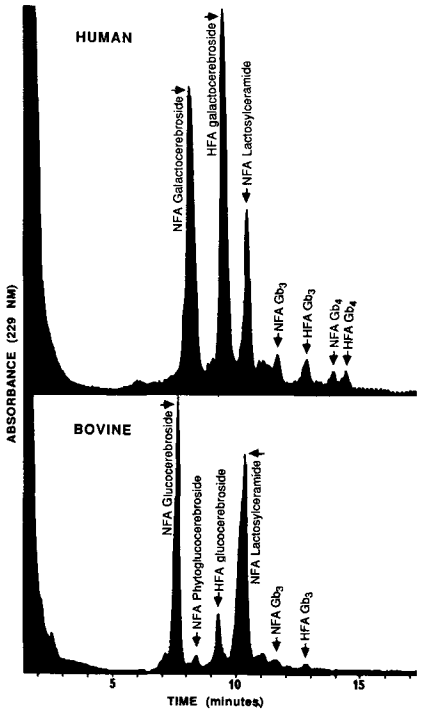

FIG. 2. A comparison of the neutral glycosphingolipid profiles of human and bovine milk by high-pressure liquid chromatography.

TABLE 1

Mean Neutral Glycosphingolipid Content of Bovine Milk[a]

Lipid	Concentration (nM)	Percent of total glycosphingolipids
GlcCer NFA[b]	7960 ± 121	29.2 ± 0.3
phyto GlcCer NFA	162 ± 10	0.59 ± 0.03
GlcCer HFA	1320 ± 86	4.9 ± 0.4
LacCer NFA	17380 ± 404	63.8 ± 0.5
LacCer HFA	420 ± 16	1.54 ± 0.06

[a]Mean ± SEM; n = 5.

[b]NFA, non-hydroxylated (normal) fatty acids.

1.3 μM, accounts for approximately 5%. HFA lactosylceramide and NFA phytoglucosylceramide are found in small amounts. Neither Gb_3 nor globoside was detected in these commercial samples. Note the lower relative standard errors for these analyses, possibly reflecting the highly pooled nature of these samples.

The glycolipids of sixteen individual samples of human milk, representing a wide range of maternal age, stage of lactation, and parity, were measured in duplicate, and the results are shown in Table 2. In this set of analyses, the HFA lactosylceramide was more fully resolved than in the chromatograms shown in Figure 2; its peak, immediately following NFA lactosylceramide, was baseline resolved, allowing unequivocal quantitation. In a separate chromatographic analysis (not shown), the NFA glucocerebroside and NFA galactocerebroside peaks were baseline resolved, and the glucocerebroside, which occurs as a shoulder of the galactocerebroside in Figure 2 and included in the total

TABLE 2

Mean Neutral Glycosphingolipid Content of Human Milk[a]

Lipid	Concentration (nM)	Percent of total glycosphingolipids
NFA[b] cerebrosides	1825 ± 170	37.6 ± 0.9
HFA[c] cerebrosides	1741 ± 163	36.6 ± 1.3
LacCer NFA	931 ± 117	18.7 ± 1.6
LacCer HFA	101 ± 15	2.4 ± 0.2
Gb_3 NFA	50 ± 8	1.1 ± 0.1
Gb_3 HFA	73 ± 11	1.6 ± 0.2
Gb_4 NFA	45 ± 6	1.0 ± 0.1
Gb_4 HFA	46 ± 6	1.1 ± 0.1

[a]Mean ± SEM; n = 16.

[b]NFA, non-hydroxylated (normal) fatty acids.

[c]HFA, hydroxylated fatty acids.

NFA cerebroside value of Table 2, was found to comprise 21% of the NFA cerebrosides. The HFA cerebrosides (glucocerebroside and galactocerebroside) always coelute in this chromatographic system. The total cerebroside peaks (NFA glucocerebroside, NFA galactocerebroside, and HFA cerebrosides) represent 75% of the lower phase neutral glycolipids of human milk. Lactosylceramide accounts for over 20% of the total. Thus, Gb_3 and Gb_4 represent a minor but consistent component of human milk. For most of the glycolipids of human milk, the NFA and HFA species are present in approximately equal proportions, except for lactosylceramide, of which 90% is the NFA species.

When the concentration of each type of human milk glycolipid was expressed as a percentage of the total glycolipids, the standard error of the mean represented by each species was approximately 10% of the mean, with the exception of the major constituents, the cerebrosides, for which the standard error was less than 4% of the mean. When expressed as absolute glycolipid concentrations in milk, the measured error was approximately 15% of the mean in most cases, except for the cerebrosides, which displayed an error of approximately 10% of the mean. The Pearson product moment correlation ($r = 0.7$) indicates that approximately half ($r^2 = 0.5$) of this variation is attributable to aspects of the analytical method, and approximately half is attributable to biological differences in the glycolipid content of the milks of individual donors.

DISCUSSION

The differences in the HPLC profiles of the neutral glycosphingolipids of whole human and of whole bovine milk are striking (Tables 1 and 2). The dominant species of bovine milk are NFA lactosylceramide and its precursor, NFA glucosylceramide. In human milk, NFA cerebrosides and HFA cerebrosides predominate; NFA lactosylceramide is the other major component. These findings are consistent with previous reports on the major glycolipids found in milk and in milk components. For example, ceramide monohexoside and ceramide dihexoside were identified in bovine milk (19). These glycolipids were isolated from bovine milk fat globules (20), and glucose was found to be the only carbohydrate in the ceramide monohexoside, thus identifying it as glucocerebroside, and

ceramide dihexoside contained both glucose and galactose, consistent with the lactosylceramide structure. The major fatty acids identified from these compounds were 16:0, 22:0 and 23:0, consistent with our finding mainly NFA glucocerebroside and NFA lactosylceramide. The glycolipids from MFGM were compared with those from skim milk for both bovine and caprine milks (12). For both of these milks, the glycolipids were of the NFA type, the milk fat globule membrane contained over half of the total glycolipids of milk, and their glycolipids contained longer chain fatty acids than those from the skim milk. This is consistent with the glycolipid patterns we found in whole bovine milk.

In a comparison of the glycolipids of human and bovine milk fat globule membrane, 88% of the monohexoslycerамide in human milk fat globule membrane was galactocerebroside, and only 12% was glucocerebroside, in contrast to bovine monohexosylceramide, which was all glucocerebroside (13). In whole human milk we found that glucocerebroside was approximately 21% of the NFA monohexosylceramide. The content of cerebrosides was reported to be approximately equal to that of lactosylceramide in bovine MFGM, whereas in human MFGM there is much more cerebroside than lactosylceramide; our results from whole milks are consistent with these findings. Also, the bovine MFGM glycolipids were reported to contain only NFA species, whereas human MFGM galactocerebroside contained 20% HFA.

Human MFGM was reported to be much higher in total glycolipids than that of bovine; however, our data from whole milks (Tables 1 and 2) indicate that the total neutral glycosphingolipid of whole bovine milk is greater than 27 μM, while the concentration in whole human milk is approximately 5 μM. We chose to compare the glycolipids of whole milk rather than of purified milk components to avoid potentially confounding effects such as differential yields of similar compartments from the milks of different species.

The data reported herein are consistent with the above reports, taking into account that glycolipids of the MFGM differ from those found in the fluid phase, and that the MFGM glycolipids represent only one component of the total glycolipids found in whole milk.

During the analysis of bovine and caprine milk polar lipids by thin-layer chromatography, the sporadic presence of a faint band corresponding to trihexoside ceramide has been reported (21). Our data confirm that Gb_3 is present infrequently, and only in very low amounts, in bovine milk samples. To our knowledge, ours is the first report to define this material as Gb_3, and to demonstrate the presence of Gb_3 and Gb_4 in human milk. These glycolipids were found in all of the human samples tested, although the amount in milk was relatively low (approximately 1% for the NFA and HFA species of each glycolipid), and somewhat variable.

Although the Gb_3 and Gb_4 levels were low relative to the mono- and dihexosides, they have the potential to be highly significant biologically. Both of these glycolipids contain the galactose α1-4 galactose structure that acts as the receptor for adhesins of pathogenic *E. coli,* and as the receptor for toxins.

The variation in measured glycolipid levels of bovine milk was quite low, consistent with the virtual elimination of individual variation due to the extremely highly pooled nature of commercial milk. In contrast, the variation in measured glycolipid levels of human milk was statistically attributed half to error in the measurement technique and half to biological variation. The lower variation seen when the data was expressed as the percent of total glycolipids, compared with the data expressed as absolute amounts per volume of milk, suggests that much of the error of the analytical method occurs during lipid extraction, isolation, and/or the absolute injection, which would affect absolute quantitation more than the relative ratios of glycolipids. The presence of significant variation among different mothers suggests biological variation in the glycolipid content of human milk, which could be related to the blood group types of the mothers, in which genetic differences in glycolipid metabolism could result in biological variation in milk glycolipids. Supporting this concept is the report that individuals of different P blood group types have different globo-series glycolipid patterns in erythrocytes, including Gb_4, Gb_3, and lactosylceramide (see structures, Fig. 1) (22). Thus, the relationship between blood group types, milk glycolipid patterns, and incidence of specific disease in infants should be the subject of further studies.

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We wish to thank Dr. Robert H. McCluer for his encouragement and advice in these studies. The technical assistance of Leslie Finck Ledsham, Edward Ullman and Jesse Lerman is gratefully acknowledged; we also thank Miriam Erikson, Director of the Central Massachusetts Human Milk Bank, Worcester, MA, for providing the human milk samples. This study was supported in part by grant HD13021 from the National Institute of Child Health and Human Development and contract #100220023SC from the Massachusetts Department of Mental Retardation.

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Oxidative Interactions of Cholesterol in the Milk Fat Globule Membrane[1]

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The effects of oxidative interactions between cholesterol and milk fat globule membrane (MFGM) components, *i.e.*, nonlipid fraction, total lipid, nonpolar lipid and polar lipid, on cholesterol oxidation were studied in the presence and absence of water. In the dry state, cholesterol natively present in MFGM appeared to be protected at 135°C. The nonpolar lipid and nonlipid fraction contributed to the protective effect of MFGM. Added cholesterol accelerated the oxidation of membrane lipid fractions. At 75°C, pure cholesterol and membrane lipid fractions did not show significant interaction. However, cholesterol and other lipids in MFGM were less stable than when these were heated separately. When cholesterol and membrane lipids were mixed in an aqueous medium at 75°C, each accelerated the oxidation of the other. The MFGM exhibited a high protective effect on cholesterol oxidation in an aqueous environment. The nonlipid fraction protected cholesterol against oxidation, whereas the lipid fraction was destructive. In the absence of water, the net balance between these two opposing factors was destructive. The presence of water reversed the balance in favor of protection.
Lipids 27, 928-932 (1992).

Bovine milk contains approximately 3-5% fat in the form of globules and these are surrounded by a thin membrane (1). This milk fat globule membrane (MFGM) consists of proteins, glycoproteins, triacylglycerols, cholesterol, enzymes and other minor components. The unsaturated lipids in the membrane were reported to play a role in initiating the oxidation of milk fat (2). Chemical and microscopical data (3-7) indicate that the MFGM originates from the plasma membrane of the secretory cells. Therefore, MFGM can be defined as a modified plasma membrane. Oxidation of membrane lipids would naturally be expected to cause alterations in membrane function which eventually lead to disturbance in cell physiology.

The objective of this work was to investigate the influence of the interaction among the membrane components on their oxidative stability using MFGM as a model membrane system.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol and silicic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol used in this study contained less than 0.9% of contaminants, including 7-ketocholesterol, 7β-hydroxycholesterol, 5,6-epoxides and cholest-3,5-dien-7-one. Cholesterol oxide standards and silylating agents were obtained from Steraloid Inc. (Wilton, NH) and Pierce Chemical Co. (Rockford, IL), respectively. Solvents were high-performance liquid chromatography (HPLC) grade from various sources.

Preparation, fractionation and characterization of MFGM. Raw milk was obtained from the University of Massachusetts farm and pasteurized at 63°C for 30 min. Cream was separated by centrifugation, then washed twice with 3 vol of Tris-HCl buffer (10 mM, pH 7.5 containing 0.25 M of sucrose and 1.0 mM of $MgCl_2$) and once with 1 vol of 50 mM Tris-HCl buffer (pH 7.5) (4). The washed cream was resuspended in 50 mM Tris-HCl buffer, and the cream structure was destroyed by freezing and thawing to liberate MFGM from the cream structure (8). Butter was separated from the MFGM suspension by centrifugation at $2,500 \times g$ for 20 min. The butter was washed with 1 vol of distilled water to recover the residual MFGM, and the water part was combined with the MFGM suspension (9). The membrane suspension was centrifuged at $100,000 \times g$ for 1 h to obtain the membrane (10). The pellets were dispersed in distilled water and quickly recentrifuged to remove the buffer salts from the membrane fraction. The membrane was then freeze-dried and stored at −40°C.

The freeze-dried MFGM was washed 3 times with 5 vol of $CHCl_3$/MeOH (2:1, vol/vol) and centrifuged at $5,000 \times g$ for 30 min to obtain the nonlipid fraction (NL) of MFGM, which was dried and stored at −40°C. Solvent in the extract was removed on a rotary evaporator to obtain crude MFGM lipid. The crude lipid was purified (11) and fractionated into polar and nonpolar fractions by silicic acid column chromatography. Chloroform and methanol were used to elute the nonpolar lipids (NPL) and the polar lipids (PL), respectively (12).

PL and NPL fractions were separated into their components by thin-layer chromatography (TLC) (silica gel G, 0.25 mm thickness). The solvent system used for NPL was petroleum hydrocarbon/diethyl ether/acetic acid (90:10:1, vol/vol/vol) (12), and that used for PL was $CHCl_3$/MeOH/water/18.4% aqueous NH_3 (130:70:8:0.5, vol/vol/vol/vol).

TLC fractions were visualized by heating the plates at 120°C for 25 min after spraying with a potassium dichromate/sulfuric acid solution. The lipid classes were quantitated with a TLC densitometer.

Sample treatment. Solutions of cholesterol and membrane lipid in chloroform (1 mg each) were placed in vials, and the solvent was evaporated on a rotary evaporator to make a thin film on the bottom of each vial. For the dry samples, the vials were placed in an oven at 135°C for 40 h or at 75°C for 40 d. For the aqueous samples, 1 mL of 10 mM Tris buffer was added to each vial and incubated at 75°C for 6 d. The lipids in the reaction tubes were extracted with 3.5 mL of chloroform/methanol (2:1, vol/vol) and divided into two parts, one for cholesterol analysis and the other for fatty acid analysis.

Cholesterol analysis. Cholesterol and its oxides were separated from the heated mixtures of cholesterol and membrane lipids by silicic acid column chromatography. The NPL, mainly triacylglycerols, were eluted with hexane/diethyl ether (95:5, vol/vol), followed by cholesterol and its oxidation products with 100% diethyl ether. Phospholipids remained on the column. The ether fraction was

[1]Based on a paper presented at the Symposium on Milk Lipids held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

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Abbreviations: BSTFA, *N,O-bis*-(trimethylsilyl)trifluoroacetamide; HPLC, high-performance liquid chromatography; MFGM, milk fat globule membrane; NL, nonlipid fraction; NPL, nonpolar lipids; PL, polar lipids; TL, total lipids; TLC, thin-layer chromatography; TMCS, trimethylchlorosilane.

collected and silylated with *N,O-bis*-(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% trimethylchlorosilane (TMCS) at 80°C for 1 h. The trimethylsilyl ethers were separated on a Varian 3700 GC (Varian Associates, Palo Alto, CA) equipped with an Ultra-1 capillary column (50 m, 0.2 mm i.d. and 0.33 μm film thickness, Hewlett-Packard, Avondale, PA) with temperature programming from 100°C to 300°C at the rate of 10°C/min. 5α-Androstan-3β-ol-17-one acetate was used as an internal standard for the analysis of cholesterol and its oxides.

Fatty acid analysis. Oxidation of triacylglycerols and free fatty acids was traced according to the loss of their fatty acyl chains. Fatty acid analysis was done by methylating the whole lipid portion directly without using silicic acid column chromatography. The lipid samples containing 1–2 mg of lipid were saponified with 0.5 mL of 2N methanolic KOH solution at 65°C for 25 min, then methylated with 0.5 mL of 14% BF_3/methanol solution at 65°C. After 25 min reaction, residual methylating agent was deactivated by adding 0.5 mL of water. The fatty acid methyl esters were extracted with hexane and analyzed by gas chromatography on a Supelcowax-10 capillary column (30 m, 0.32 mm i.d. and 0.25 μm film thickness, Supelco Inc., Bellefonte, PA). Triheptadecanoin was used as an internal standard. The graphs in this report represent the averages of triplicate analyses.

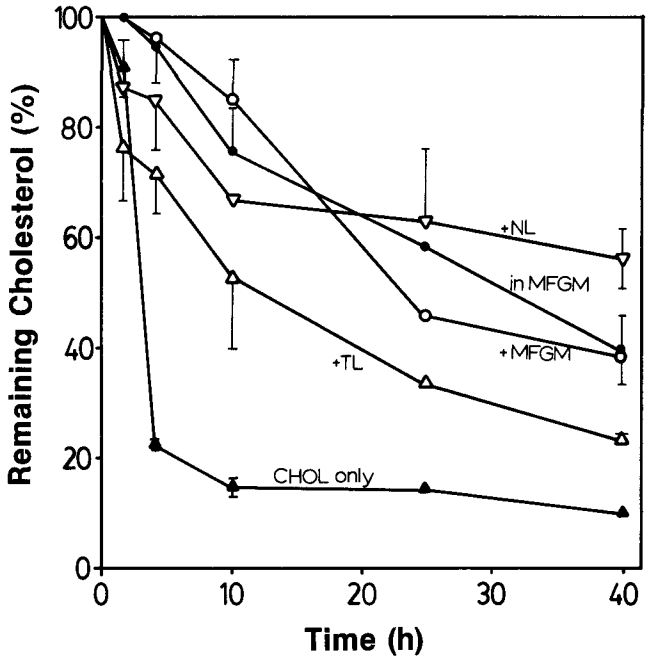

FIG. 1. Effect of membrane components on cholesterol oxidation in the dry state at 135°C. MFGM, milk fat globule membrane; NL, nonlipids; TL, total lipids; CHOL, cholesterol.

RESULTS

Composition of milk fat globule membrane. The water content of the dried MFGM was 7.6% (w/w). The MFGM solids, 92.4%, consisted of 59.7% total lipids (TL) and 32.7% NL. Spectrophotometric analysis of the protein in MFGM using bovine serum albumin as a standard showed a total protein content of 32.0% (w/w), indicating that 98% of the nonlipid fraction was protein. TL were fractionated into NPL and PL on a silicic acid column. The NPL were eluted first with chloroform followed by PL with methanol. The NPL and PL in the dried MFGM accounted for 46.6% and 13.1%, respectively.

The NPL contained mainly triacylglycerols (42.5%), diacylglycerols (32.0%) and free fatty acids (6.3%). The PL contained 17.5% phospholipids (29.7% sphingomyelin, 24.8% phosphatidylcholine, 17.4% phosphatidylethanolamine, 13.3% phosphatidylinositol and 14.8% others) and 82.5% of unidentified compounds, probably glycerides. The lipid fractions (TL, NPL and PL) did not show significant differences in their fatty acid profiles (38.6–41.4% palmitate, 10.3–17.8% stearate, 12.6–15.5% oleate, etc.).

Effect of MFGM and its components on cholesterol oxidation in the dry state. At 135°C, cholesterol in the MFGM appears to be protected against oxidation when compared to pure cholesterol (Fig. 1). The TL fraction inhibited cholesterol destruction. The NL fraction of MFGM was more protective than the TL fraction. When the whole MFGM (containing 32.7% NL and 59.7% TL) was added to pure cholesterol, no changes in cholesterol amount were observed until 5 h heating at 135°C, when it started to decrease. Finally, the remaining cholesterol reached a level in between those produced by NL and TL (Fig. 1). The added MFGM showed a net protective effect on cholesterol oxidation.

When the PL fraction of MFGM was added to cholesterol at 135°C, it did not influence the stability of cholesterol (Fig. 2). The NPL protected cholesterol against oxidation. The effect of TL (containing 21.9% PL and 78.1% NPL) on cholesterol oxidation was in between the effects of its two components, *i.e.*, polar and nonpolar.

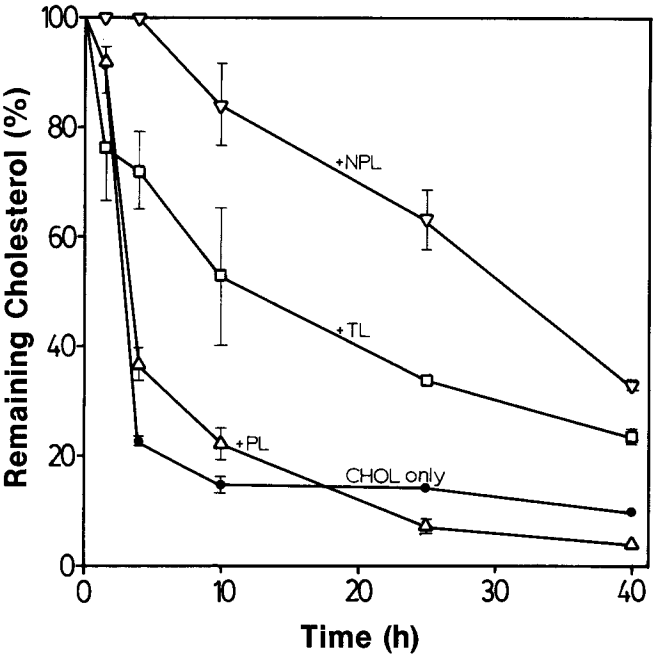

FIG. 2. Effect of membrane lipid fractions on cholesterol oxidation in the dry state at 135°C. TL, total lipids; NPL, nonpolar lipids; PL, polar lipids; CHOL, cholesterol.

At 75°C, cholesterol in MFGM was much less stable than pure cholesterol (Fig. 3). Solid cholesterol is very

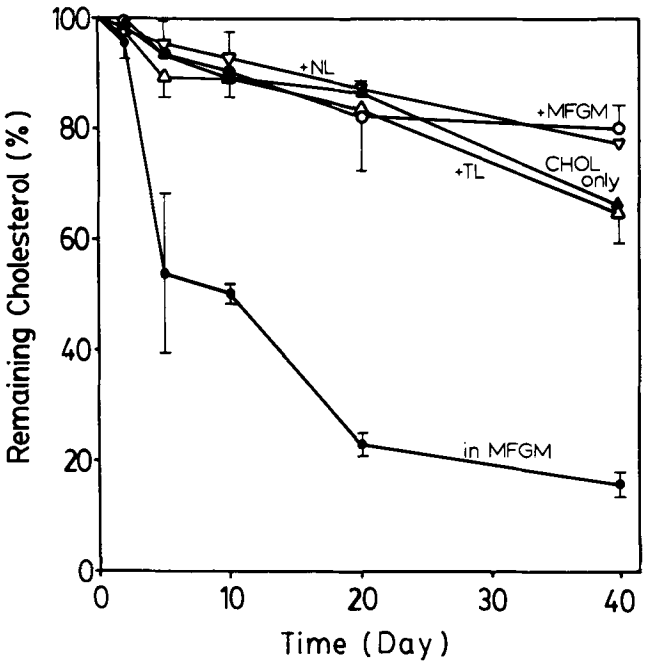

FIG. 3. Effect of membrane components on cholesterol oxidation in the dry state at 75°C. MFGM, milk fat globule membrane; NL, nonlipids; TL, total lipids; CHOL, cholesterol.

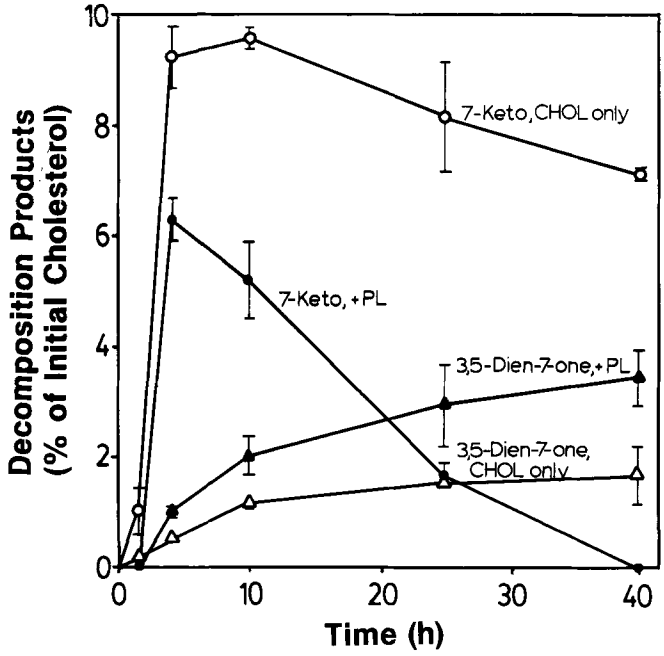

FIG. 4. Effect of membrane polar lipids (PL) on the production of 7-ketocholesterol (7-Keto) and cholesta-3,5-dien-7-one (3,5-Dien-7-one) in the dry state at 135°C.

stable at low temperature. Molecular orientation or the surrounding components in MFGM may have accelerated cholesterol oxidation. Addition of MFGM, TL or NL did not show a significant effect on cholesterol oxidation at 75°C. Only a slight protective effect of added NL and MFGM was observed after 40 d of heating (Fig. 3). The lack of an effect of added MFGM and its components on cholesterol oxidation at 75°C may be due to the lack of interaction between cholesterol and these components. When the solvent was evaporated during sample preparation, cholesterol was crystallized and separated from the other components added. Therefore, at 75°C the opportunity for interaction was minimized, whereas at 135°C, the cholesterol crystals were melted and well mixed. The difference in cholesterol stability between the mixtures and dry MFGM at 75°C appears to reflect the effectiveness of the natural mixing in MFGM.

In the presence of MFGM and its components, cholesterol produced very low levels of oxides, although substrate cholesterol was decreased during heating. Only when the PL fraction was added did cholesterol produce relatively large amounts of 7-ketocholesterol and cholesta-3,5-dien-7-one (Fig. 4). Since the PL fraction of MFGM contains phosphatidylcholine and sphingomyelin as the main components, the oxide profile produced was similar to that of phosphatidylcholine and sphingomyelin, *i.e.*, formation of 7-ketocholesterol at the beginning of heating followed by its conversion to cholesta-3,5-dien-7-one.

Effect of MFGM and its components on cholesterol oxidation in aqueous system. As demonstrated in Figure 5, MFGM cholesterol was very stable during six days of incubation in buffer at 75°C. Sonication of the MFGM to destroy or reorganize its native structure did not affect the stability of cholesterol in the membrane. When MFGM was added to pure cholesterol, it exhibited a

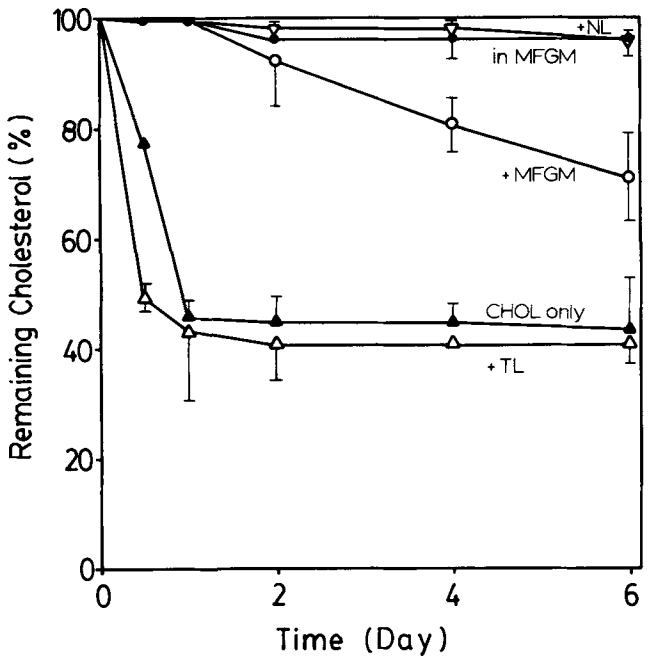

FIG. 5. Effect of membrane components on cholesterol oxidation in dispersion at 75°C, pH 7.4. MFGM, milk fat globule membrane; NL, nonlipids; TL, total lipids; CHOL, cholesterol.

protective effect. In contrast to the dry state, in aqueous dispersion the lipid portion of MFGM (TL, NPL and PL) accelerated cholesterol oxidation at the beginning of incubation, *i.e.*, the first 12 h of heating. The stability of cholesterol in the presence of NPL or PL was similar to that in the presence of TL (Fig. 5). The NL fraction

appears to be the most protective among the MFGM components tested.

In the presence of whole membrane materials or NL fraction of MFGM, cholesterol did not produce appreciable amounts of cholesterol oxides. However, in the presence of the lipid portion (TL, NPL or PL) of MFGM, approximately the same level of total oxides was produced as that observed in the pure cholesterol control. Higher amounts of epoxides and 7-hydroxycholesterols also were observed when the MFGM lipid was added. The lipid fraction favored 7-hydroxylation but did not have a significant effect on the β- to α-form ratios of 7-hydroxycholesterols and epoxides.

Effect of added cholesterol on the oxidation of membrane lipid. The saturated fatty acids exhibited higher stability than the unsaturated ones in dry MFGM heated at 135°C, and in MFGM, TL, PL and NPL heated at 75°C in the presence of water. However, saturated short chains in the lipid fractions (TL, PL and NPL) of MFGM showed low stability when heated at 135°C in the dry state. Figure 6 shows that the short chain fatty acids were less stable than the longer chains, both saturated and unsaturated. An explanation for the more rapid disappearance of the short chains cannot be provided at this time.

When cholesterol was added to MFGM or its lipid fractions at 135°C, it accelerated the destruction of the C_{18} chains, *i.e.,* stearate, oleate and linolenate, of membrane lipid, but the short chain fatty acids became more stable. The destructive effect of cholesterol on the oxidation of C_{18} chains was much greater when it was added to membrane PL (Fig. 7).

In contrast to the behavior at 135°C, addition of cholesterol to MFGM did not affect the stability of membrane lipids at 75°C. As discussed earlier, at 75°C in the dry state, cholesterol and added components did not appear to interact.

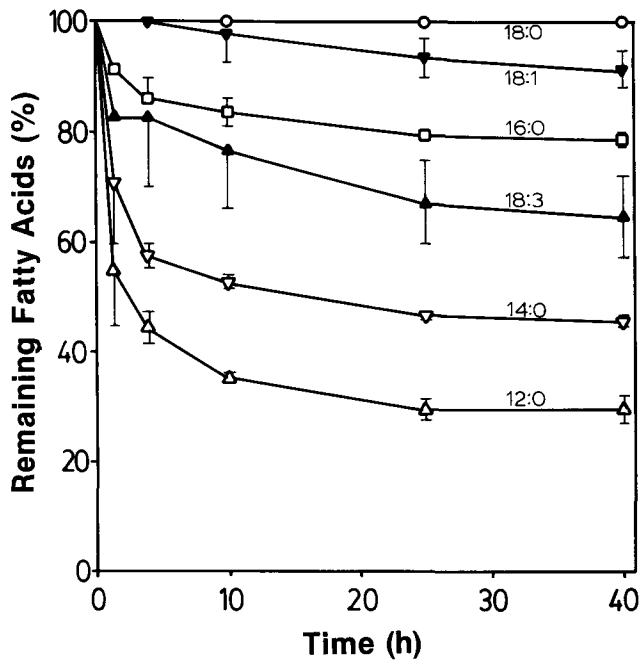

FIG. 6. Stability of fatty acyl chains in membrane total lipid fraction during heating in the dry state at 135°C.

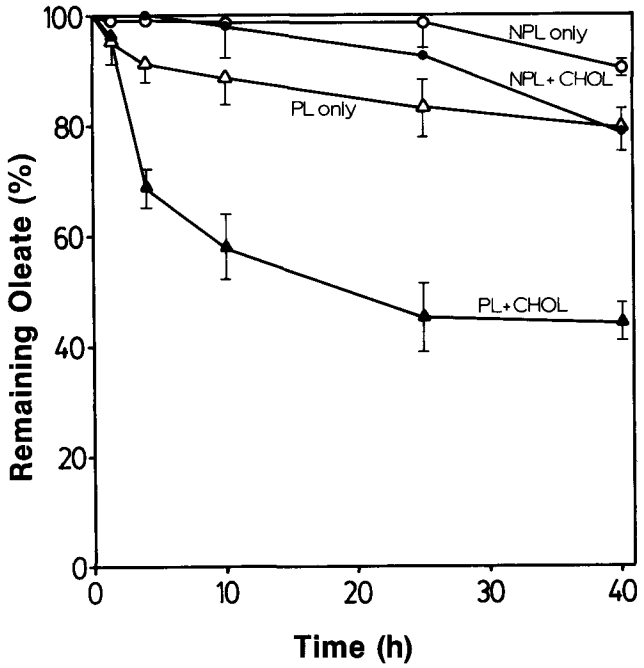

FIG. 7. Effect of cholesterol on the oxidation of oleate in membrane lipids at 135°C. NPL, nonpolar lipids; PL, polar lipids; CHOL, cholesterol.

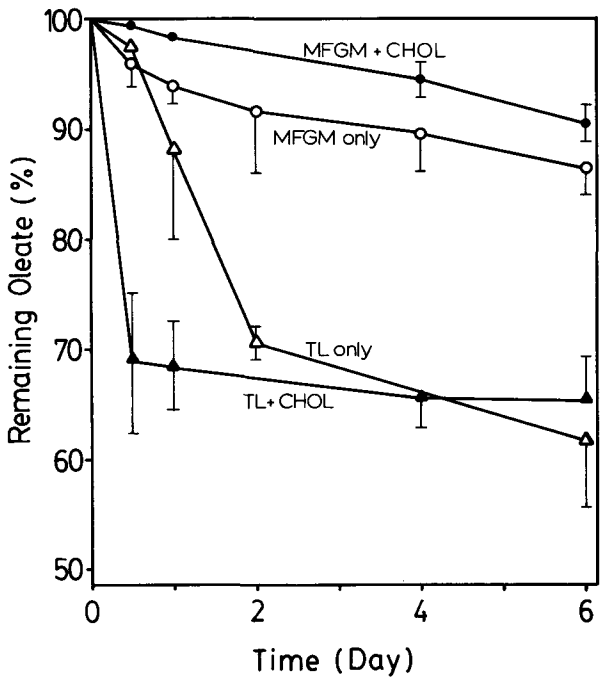

FIG. 8. Effect of cholesterol on the oxidation of oleate in membrane lipids in dispersion at 75°C, pH 7.4. MFGM, milk fat globule membrane; TL, total lipids; CHOL, cholesterol.

The lipids in MFGM were very stable in aqueous dispersions when compared to each of its lipid fractions incubated alone (Fig. 8). Stability of PL and NPL in the absence or presence of cholesterol also showed similar patterns to those of TL. When cholesterol and whole MFGM

were mixed in aqueous dispersion, they protected each other from oxidation. However, when cholesterol was mixed with the MFGM lipid portion, they accelerated each other's oxidation at the beginning of incubation and then reached a plateau (Figs. 5 and 8).

DISCUSSION

The MFGM consists of various components, such as proteins, triacylglycerols, phospholipids and other compounds, including monoacylglycerols, diacylglycerols and free fatty acids.

In the dry state at 135°C, the effects of added membrane components on cholesterol oxidation and the oxidation of cholesterol natively present in MFGM showed consistent results, *i.e.,* MFGM and its components protected cholesterol from oxidation, and cholesterol accelerated the oxidation of membrane lipids. However, because of poor interaction between cholesterol and added MFGM components at 75°C, only cholesterol in MFGM showed a significant difference from the cholesterol control. Cholesterol natively present in MFGM is well surrounded by other membrane components. The interactions between cholesterol and neighboring molecules, and perhaps their physical arrangement, appears to have accelerated cholesterol oxidation. When the heating temperature was increased from 75 to 135°C, the stability of pure cholesterol changed drastically whereas in MFGM, the interaction between cholesterol and neighboring molecules counteracted the temperature effect on the stability of cholesterol. Consequently, the neighboring components in MFGM which are destructive at 75°C showed apparent protection at 135°C.

When incubated alone, cholesterol oxidized faster in aqueous dispersion than in the dry state at the same temperature (Figs. 3 and 5). However, cholesterol natively present in MFGM exhibited high stability in aqueous dispersion. The lipid portion of MFGM (TL, NPL, PL) accelerated cholesterol oxidation at the beginning of incubation, then did not show a significant effect on further cholesterol oxidation in the aqueous system.

Added cholesterol accelerated the oxidation of MFGM lipid fractions (TL, NPL, PL) in both dry and aqueous systems, and it also promoted oxidation of the lipids in whole MFGM in the dry state. However, in the aqueous system, added cholesterol protected the lipids in MFGM against oxidation. Cholesterol tended to accelerate the oxidation of the other lipids in the membrane. However, in the presence of water, the NL fraction of MFGM appeared to provide a condition where both cholesterol and the other lipid components protected each other (Table 1).

The MFGM exhibited a high protective effect on cholesterol oxidation in an aqueous environment. Its NL fraction protects cholesterol from oxidation, while the lipid fraction was destructive in the early stages of cholesterol oxidation. In the absence of water, the net balance between these two opposing factors was destructive. The presence of water tipped the balance in favor of protection.

The results of this study suggest that both biochemical and physico-chemical factors are important in protecting the membrane against destruction.

TABLE 1

Interactions Between Cholesterol and Other Milk Fat Globule Membrane Components[a]

	Dry				Aqueous	
	135°C		75°C		75°C	
	C	L	C	L	C	L
Individual mixing	−	+	=	=	+	+
In membrane	−	+	+	+	−	−

[a]C, cholesterol; L, membrane lipid; −, oxidation decreased relative to control; +, oxidation increased; =, no difference.

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High-Performance Liquid Chromatography of Human Milk Triacylglycerols and Gas Chromatography of Component Fatty Acids[1]

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Human milk triacylglycerols were separated by high-performance liquid chromatography. A 5-μ Supelcosil LC-18 column (Supelco, Inc., Bellefonte, PA) was used with acetone/acetonitrile (64:36, vol/vol) as mobile phase. Triacylglycerols were tentatively identified based on theoretical carbon number and relative retention time. Despite changes resulting from dietary fat variation, the major component triacylglycerols were those composed of palmitic, oleic and linoleic acids. Triacylglycerols with palmitic, stearic and oleic acids were present as minor components. Fatty acids were quantified by gas chromatography relative to an internal standard. Ratios of n-6/n-3 fatty acids were found to be higher than previously reported. *Lipids 27*, **933-939 (1992).**

The lipids contained in human milk are derived from maternal depot fat stores, endogenous synthesis in the mammary gland and dietary sources. Several studies have demonstrated the relationship between maternal dietary intake and milk fatty acid composition and have shown a dietary effect on both mobilization of fats from body stores and endogenous synthesis. Insull *et al.* (1) were the first to show that human milk lipids are affected by maternal diet: when on a high fat diet, the women studied produced milk high in triacylglycerols of long-chain fatty acids, reflecting the fatty acid composition of the dietary fats and low endogenous fatty acid synthesis; when on a low fat, calorie-restricted diet, the milk predominated in triacylglycerols of long-chain fatty acids derived from body fat sores; upon ingesting a very low fat diet, the milk showed a marked increase in medium-chain fatty acids indicating increased endogenous synthesis. In agreement with the principles proposed by Insull *et al.* (1), studies on Gambian women (2), Egyptian women (3) and American vegetarian women (4) have shown that milk fat content mirrors maternal dietary fat intake.

While the component fatty acids of human milk are well documented (4-7), only a few studies have investigated the triacylglycerol composition of human milk (8,9). Because triacylglycerols represent approximately 98% of the lipids of human milk (7), the availability of a method for their analysis would facilitate a greater understanding of the direct effect of maternal diet on milk fat composition and structure. To this end, we have developed a procedure for the separation and identification of triacylglycerols by high-performance liquid chromatography (HPLC).

HPLC of naturally occurring lipids has been extensively used, and the sequence of triacylglycerol elution in the present system has been established (10). Using this method, triacylglycerols were identified by their equivalent carbon numbers (ECN), which takes into account the carbon numbers and number of double bonds of the various acyl groups. Identification of triacylglycerols was further verified by calculating retention times relative to triolein and by comparing these retention times with those of known standard triacylglycerols, as well as by further analysis of component peaks (10).

The fatty acids composing human milk triacylglycerols were quantified by gas chromatography relative to an internal standard. The content of long-chain polyunsaturated fatty acids (LC PUFA; C_{18} and greater) in milk could thus be accurately quantified. The very low concentrations of LC PUFA in human milk should not be taken as an indication of their unimportance in infant nutrition (11-14). Carlson *et al.* (11) observed that premature infants fed human milk containing docosahexaenoic acid (DHA, 22:6n-3) had significantly higher concentrations of DHA in red blood cell (RBC) membrane phospholipids than infants fed milk formulas lacking DHA. Using nonhuman primates, Neuringer *et al.* (13) have presented evidence implicating the n-3 fatty acids, particularly DHA, in critical neural and retinal development in the neonate. DHA appears to be preferentially incorporated into brain and retinal phospholipids (15), and the high level of LC PUFA in these phospholipids seems to contribute to the characteristic membrane fluidity observed in these tissues (16-18); it also appears essential to their proper functioning (19). The data by Anderson *et al.* (12) suggest that DHA is the optimal n-3 fatty acid for brain and retinal development; yet current infant formulas do not provide this long-chain fatty acid nor its precursor, eicosapentaenoic acid (EPA, 20:5n-3).

EXPERIMENTAL PROCEDURES

Materials. The instrument used for triacylglycerol analyses consisted of a Waters 501 chromatographic pump (Waters, Inc., Milton, MA), a Rheodyne loop injector (20 μL, Rheodyne Model 7120, Berkeley, CA), and a Waters Model 410 differential refractometer detector; a Hewlett-Packard (Palto Alto, CA) Model 3390A electronic integrator was used to record separations and retention times. Triacylglycerols were separated on a 25 cm × 4.6 mm i.d. column packed with Supelcosil LC-18 (5 micron particle size, Supelco, Inc., Bellefonte, PA).

A Hewlett-Packard Model 5890 gas chromatograph

[1]Based on a paper presented at the Symposium on Milk Lipids held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

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Abbreviations: AA, arachidonic acid (20:4n-6); CN, carbon number; CV, coefficient of variation; D, decanoic acid (10:0); DHA, docosahexaenoic acid (22:6n-3); ECN, equivalent carbon number; EPA, eicosapentaenoic acid (20:5n-3); FA, fatty acid(s); FAME, fatty acid methyl ester(s); GC, gas chromatography; HPLC, high-performance liquid chromatography; L, lauric acid (12:0); LC PUFA, long-chain polyunsaturated fatty acid(s); Ln, linolenic acid (18:3n-3); Lo, linoleic acid (18:2n-6); M, myristic acid (14:0); O, oleic acid (18:1n-9); P, palmitic acid (16:0); Po, palmitoleic acid (16:1n-7); TAG, triacylglycerol; S, stearic acid (18:0); RBC, red blood cell(s); TCN, theoretical carbon number; TLC, thin-layer chromatography.

equipped with a Supelcowax 10 column (30 m × 0.25 mm i.d.) (Supelco, Inc.) of film thickness 0.25 microns was used for fatty acid analyses. The column conditions were: temperature, 180°C; injector 250°C; detector 270°C; linear velocity, 44.6 cm/s. Sample size for injection was approximately 2 μL containing 0.0094–0.0099 μg ester. Separations and retention times were recorded on a Hewlett-Packard Model 3392A electronic integrator.

Reagents and standards. A mixture of HPLC grade acetone/acetonitrile (64:36, vol/vol) was used as mobile phase for HPLC analysis. Triacylglycerol standards were purchased from Nu-Chek-Prep, Inc. (Elysian, MN).

n-Octacosane was purchased from K and K Laboratories, Inc. (Plainview, NY) for use as an internal standard in fatty acid analysis. Standard PUFA methyl esters were obtained from Nu-Chek Prep, Inc.

Sample collection and preparation. Human milk was collected at 4, 6 and 8 wk *postpartum* (± 3 days) by electric pump expression of the entire contents of one breast from healthy women residing in Champaign-Urbana, IL. All samples were collected between 8 a.m. and 11 a.m., placed on ice, and transferred to the laboratory for immediate extraction.

The fat portion of a 20-mL aliquot of a milk sample was separated by centrifugation at 10,000 × *g* for 20 min at 0°C, using a Beckman Model J2-21 centrifuge (Beckman Instruments, Fullerton, CA). The lipid layer was suspended in *ca.* 100 mL of chloroform/methanol (2:1, vol/vol) for 5 min and gravity-filtered; the extract was then concentrated to approximately 2 mL with a rotary evaporator and further evaporated to dryness under N_2. The triacylglycerols were subsequently extracted with 3 mL hexane. Solid-phase extraction chromatography of the extracted lipid on silicic acid was accomplished using 6 mL Supelclean LC-Si SPE tubes (Supelco, Inc.). Subsequent elution with chloroform yielded the triacylglycerol fraction and elution with chloroform/methanol the polar fraction, following the method of Juaneda and Rocquelin (19). Purity of the triacylglycerol fraction was verified by thin-layer chromatography (TLC) on silica gel (20), and was found to be >98% triacylglycerol. Samples in chloroform were stored for a short time at −20°C in glass vials with Teflon-lined caps. Triacylglycerol solutions were prepared for HPLC by dissolving a known quantity of the triacylglycerol fraction in the mobile phase at a concentration of 10 mg/mL.

Fatty acid methyl esters (FAME) were prepared from aliquots of the triacylglycerol fractions using the AOCS Official Method Ce 2-66 (21). To measure absolute fatty acid content, octacosane was added as an internal standard because it did not coelute with any of the fatty acids. Standard FAME mixtures were cochromatographed to verify sample retention times and identify the methyl esters. The weight of each fatty acid was calculated from the equation:

$$\text{wt of FA} = \frac{\text{(peak area of FA)}}{\text{(peak area of standard)}} \times \text{(wt of standard added)} \quad [1]$$

Triacylglycerol identification[2]. The separation of the triacylglycerols in our studies was found to follow that predicted by El-Hamdy and Perkins (10). Elution progresses from the component with the lowest ECN to the highest and within an ECN from the highest degree of unsaturation to the lowest (Fig. 1). Triacylglycerol identification was based on a scheme outlined by El-Hamdy and Perkins (10): critical pairs are structures with an equivalent carbon number, defined as:

$$\text{ECN} = \text{CN} - 2n \quad [2]$$

where CN = actual carbon number; n = number of double bonds per molecule.

Triacylglycerols elute in a pattern dependent on solvent

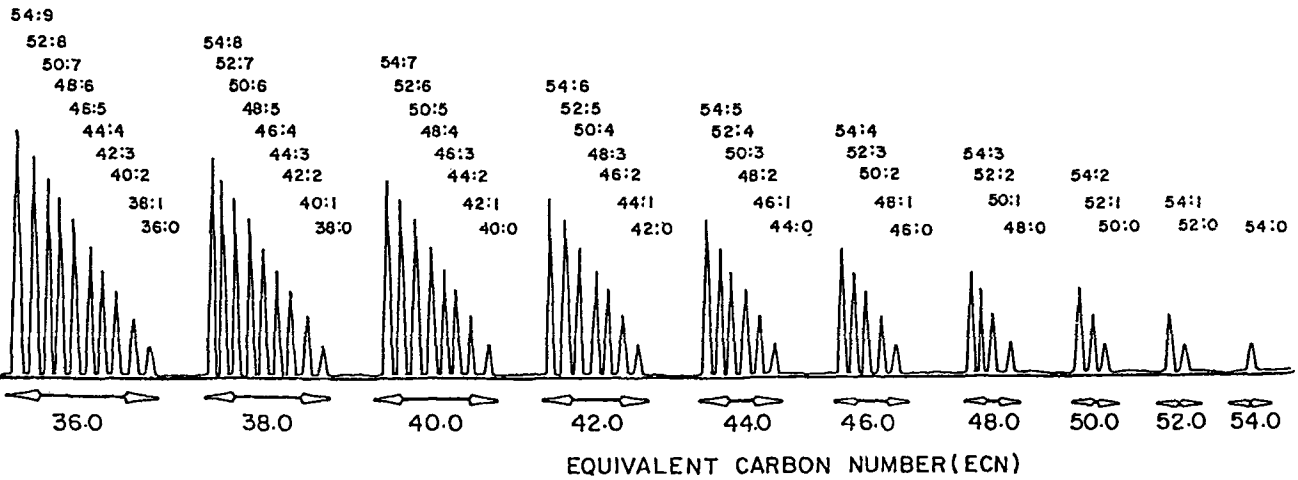

FIG. 1. Elution of triacylglycerols. Theoretical separation pattern by high-performance liquid chromatography (40).

[2]Triacylglycerol abbreviations used (*e.g.* PLO) are intended to define composition rather than positional fatty acid distribution; *i.e.*, a peak identified as PLO may be composed of the triacylglycerolisomers PLO, POL, LOP, LPO, OLP and OPL.

TABLE 1

Theoretical Carbon Numbers (TCN) and Triacylglycerols[a]

Triacylglycerol	ECN[b]	Number of carbons	Number of double bonds	TCN[c]
LnLnLn	36	54	9	33.57
LnLnPn	36	52	8	33.75
LnLnM	36	50	7	33.96
LnLnL	36	48	6	34.38
LnLoD	36	46	5	34.44
LnLoD	36	44	4	34.57
LnLL	36	42	3	35.19
LoLD	36	40	2	35.25
PoLD	36	38	1	35.37
LLL	36	36	0	36.00
LnLnLo	38	54	8	35.63
LnLnPo	38	52	7	35.75
LnLnM	38	50	6	36.38
LnLoL	38	48	5	36.44
LoLoD	38	46	4	36.50
LnDD	38	46	4	36.57
LnLM	38	44	3	37.19
LoLL	38	44	2	39.25
LoMD	38	44	2	39.25
LOD	38	40	1	37.37
PoLL	38	40	1	37.37
MLL	38	38	0	38.00
MMD	38	38	0	38.00
PLD	38	38	0	38.00
SDD	38	38	0	38.00
LoLoLn	40	54	7	37.69
LnLnO	40	54	7	37.75
LnLnP	40	52	6	38.38
LnLoM	40	50	5	38.44
LoLoL	40	48	4	38.50
LnOL	40	48	4	38.56
LoOL	40	46	3	38.62
LnMM	40	46	3	39.19
LoML	40	44	2	39.25
OLL	40	42	1	39.37
MML	40	40	0	40.00
LoLoLo	42	54	6	39.75
LoLnO	42	54	6	39.81
LnLnS	42	54	6	40.48
LnLoP	42	52	5	40.44
LoLoM	42	50	4	40.50
LnOM	42	50	4	40.56
LoOL	42	48	3	40.62
LnPM	42	48	3	41.19
LoMM	42	46	2	43.19
LoPL	42	46	2	43.25
OML	42	44	1	41.37
MMM	42	42	0	42.00
PML	52	42	0	42.00
SLL	42	42	0	42.00
LoLoO	44	54	5	41.88
LnOO	44	54	5	41.94
SLoLn	44	54	5	42.44
LoLoPn	44	52	4	42.50
LnOP	44	52	4	42.56
LoOM	44	50	3	42.65
LnPP	44	50	3	43.19
POO	44	48	2	42.75
LoMP	44	48	2	43.25
OMM	44	46	1	43.37
OPL	44	46	1	43.37
PPL	44	44	0	44.00
SML	44	44	0	44.00
PMM	44	44	0	44.00
LoLoS	46	54	4	44.50
LnOS	46	54	4	44.56
LoOP	46	52	3	44.62
LnSP	46	52	3	45.19
OMM	46	50	2	44.95
LoPP	46	50	2	45.25
OPM	46	48	1	45.38
OSL	46	48	1	45.38
PPM	46	46	0	46.00
SPL	46	46	0	46.00
OOO	48	54	3	46.12
SOLo	48	54	3	46.62
SSLn	48	54	3	47.19
OOP	48	52	2	46.75
SLoP	48	52	2	47.26
PPO	48	50	1	47.37
PPP	48	48	0	48.00
SOO	50	54	2	48.75
SSLo	50	54	2	49.25
SPO	50	52	1	49.37
SPP	50	50	0	50.00
SSO	52	54	1	51.37
SSP	52	52	0	52.00
SSS	54	54	0	54.00

[a]Triacylglycerol components as defined in footnote 2.

[b]Equivalent carbon number (ECN) is defined as ECN = CN − 2n where CN equals the actual carbon number and n equals the number of double bonds per molecule.

[c]Theoretical carbon number (TCN) is defined as: TCN = ECN − (Σ_1^3 U_i), where TCN = theoretical carbon number; ECN = equivalent carbon number; U_i = a factor determined experimentally for several fatty acids and found to be: 0.6 for oleoyl, 0.7 for linoleoyl, 0.2 for elaidoyl groups, and 0.0 for saturated acyl groups; (Σ_1^3 U_i) = the total U_i of component fatty acids present in a particular triacylglycerol (11).

composition and resolution of the column. Critical pairs may co-elute because of their close ECN values, if the column resolution is less than adequate. Among components of identical ECN, triacylglycerols containing more saturated acyl groups will tend to elute more slowly because of their higher affinity with the column packing (10). Conversely, the triacylglycerols with more unsaturated acyl groups will elute more rapidly because of decreased affinity with the stationary phase. Thus their order of elution may be accurately predicted (10).

Furthermore, the theoretical carbon numbers of all possible triacylglycerols within an essential carbon number group may be used to predict the order of elution of critical pairs of triacylglycerols (Table 1). The theoretical carbon number can be calculated by the following formula:

$$TCN = ECN - (\Sigma_1^3 U_i) \quad [3]$$

where TCN = theoretical carbon number; ECN = equivalent carbon number; U_i = a factor determined experimentally for several fatty acids and found to be: 0.6 for oleoyl, 0.7 for linoleoyl, 0.2 for elaidoyl groups, and 0.0 for saturated acyl groups; Σ_1^3 U_i = the total U_i of component fatty acids present in a particular triacylglycerol (10). Differences between TCN values indicate the possibility of separating the triacylglycerols. Some triacylglyerols, however, will have identical TCN values and cannot be separated in this system. For example, the TCN of OMM = 43.37 and the TCN of OPL = 43.37, therefore OMM

and OPL cannot be distinguished by this method, although identification may be accomplished by examining fatty acid composition of the factions.

Additionally, peaks were identified by retention time relative to triolein. To calculate relative retention times, triolein was added to mixtures for which the triacylglycerol components are known (*e.g.*, corn oil, olive oil, beef tallow) and to standard mixtures of triacylglycerols.

RESULTS AND DISCUSSION

Triacylglycerols. The separation of human milk triacylglycerols is illustrated in Figure 2. The chromatographic patterns varied slightly between subjects and with the stage of lactation; however, relative retention times were consistent among all milk samples. If a peak could not be positively identified because of co-elution with other triacylglycerols of identical TCN, the fatty acid composition of the sample was examined. Thus, if a peak could be identified as LLM, MMD, PLD or SDD (all of TCN = 38.00), and the fatty acid data revealed only trace amounts of decanoic acid, the fraction could be reliably identified as LLM. In all cases of multiple assignment choices, the triacylglycerol most closely reflecting the sample fatty acid composition was assigned to the peak.

Human milk was shown by this method to contain more than twenty-seven triacylglycerol structures, of which six, LPP, PPO, LoLoP, SSL, LSO and LoPL, accounted for 55% of the total; the remaining triacylglycerols contributed less than 5% each. The average triacylglycerol composition of human milk is shown in Table 2, representing milk samples from ten women collected at three time periods each. The data are also expressed as average percent by stage of lactation. Considerable variation existed among women, reflecting the sensitivity of milk fat to dietary fat intake. Although some variation was seen between time periods, no significant variation ($P > 0.05$) in triacylglycerol content was noted at various stages of lactation at 4, 6 and 8 wk. The data for the six most predominant triacylglycerols are shown in Figure 3 as average percentages at 4, 6 and 8 wk.

A more complex elution pattern was obtained when two columns were fixed in series (Fig. 4). Although greater resolution was obtained, more than 90 min was required to effect a separation. The increased resolution resulted in separation within critical pairs, creating shoulders on the elution peaks. This made identification and quantification considerably more difficult. Therefore the data obtained on a single column, as shown in Figure 2, were used to assess the composition of the major triacylglycerols.

Fatty acids. Palmitic, stearic, oleic and linoleic acids were the most abundant, constituting approximately 76% of the total fatty acids. Fatty acid composition was observed to differ among subjects and between time periods, but the differences were not statistically significant. Fatty acid data are compiled in Table 3.

Unlike fat from sources such as cow's milk-based formulas, the fat of human milk is very efficiently adsorbed by both the term and the preterm neonate; this is due in part to the structure of the human milk triacylglycerols (22). The distribution of fatty acids within the triacylglycerols of human milk is not random, as certain fatty acids are preferentially esterified at the *sn*-2 position (23). When

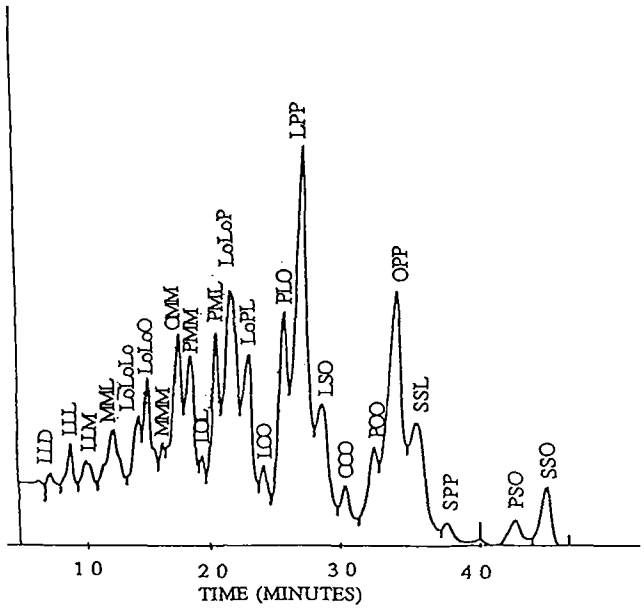

FIG. 2. Separation of human milk triacylglycerols by high-performance liquid chromatography using a 5-μ Supelcosil LC-18 silica column with a mobile phase of acetone/acetonitrile (64:36, vol/vol).

TABLE 2

Human Milk Triacylglycerols[a]

TAG[b]	Week 4		Week 6		Week 8		Total
	Area %[c]	CV[d]	Area %	CV	Area %	CV	area %[e]
LLD	0.47	0.10	0.37	0.08	0.57	0.13	0.48
LLL	1.02	0.05	1.85	0.10	1.22	0.06	1.34
LLM	0.85	0.07	0.97	0.05	1.18	0.08	1.01
MML	2.24	0.05	2.58	0.05	2.95	0.06	2.62
LoLoLo	1.14	0.08	1.68	0.02	1.82	0.05	1.57
LoLoO	2.79	0.06	3.33	0.04	3.62	0.04	3.27
MMM	0.98	0.11	2.49	0.07	1.15	0.09	1.49
OMM	4.34	0.03	4.69	0.02	5.00	0.02	4.70
PMM	3.94	0.06	4.76	0.05	4.67	0.06	4.47
LOL	1.41	0.06	1.68	0.07	1.39	0.05	1.48
PML	3.16	0.03	4.20	0.05	3.55	0.04	3.62
LoLoP	8.30	0.02	8.73	0.02	9.34	0.01	8.84
LoPL	5.63	0.04	6.10	0.03	6.40	0.02	6.07
LOO	1.72	0.03	2.28	0.05	1.99	0.02	1.99
PLO	4.31	0.03	5.53	0.06	4.74	0.03	4.84
LPP	15.03	0.02	3.76	0.02	15.12	0.02	14.69
LSO	6.55	0.04	5.93	0.03	6.15	0.01	6.21
OOO	2.74	0.05	2.02	0.02	1.86	0.02	2.18
POO	3.58	0.04	3.46	0.07	2.63	0.03	3.17
SOM	0.00	0.00	0.87	0.30	0.00	0.00	0.26
PPO	13.64	10.02	0.34	0.04	11.85	0.02	11.96
SSL	8.46	0.03	6.79	0.03	6.95	0.02	7.38
PPS	1.71	0.09	0.90	0.07	0.93	0.05	1.16
SOO	0.14	0.18	0.11	0.13	0.03	0.23	0.09
PSO	1.55	0.07	1.02	0.05	1.19	0.05	1.25
SSO	3.91	0.04	3.25	0.04	3.21	0.04	3.44
SSS	0.40	0.22	0.34	0.17	0.47	0.19	0.41

[a]Data represent area percent of triacylglycerol identified *via* high-performance liquid chromatography; identification was based on theoretical carbon number and retention time relative to triolein.
[b]Denotes triacylglycerol.
[c]Mean of 10 samples.
[d]CV denotes coefficient of variation.
[e]Mean of 30 samples.

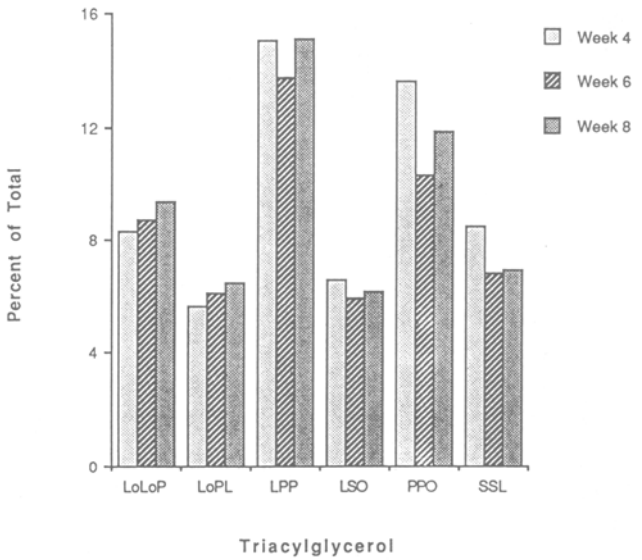

FIG. 3. Distribution of major triacylglycerols identified in human milk. Each bar represents mean of 10 samples. No significant differences were observed between time periods. Triacylglycerol components as defined in footnote 2.

Freeman *et al.* (24) hydrolyzed total human milk triacylglycerols with pancreatic lipase, they observed that 74 mol% of the palmitic acid was esterified at the *sn*-2 position. Breckenridge *et al.* (8) also observed that most of the palmitic acid was in the *sn*-2 position of the triacylglycerol moiety. After hydrolysis of the 1- and 3-position by specific lipases (such as lingual and pancreatic lipases), much of the remaining 2-palmitoylglycerol may be absorbed by the neonate. Alternatively, when palmitic acid is esterified in the *sn*-1 or *sn*-3 position, as it is in cow's milk, and subsequently released upon hydrolysis as free fatty acid, it would become susceptible to precipitation by calcium and thus excreted, resulting in the loss to the neonate of both energy and calcium (25). Oleic acid, typically the most abundant fatty acid found in milk of women on Western diets (4–7), is also present in human milk triacylglycerols in a characteristic pattern. Kuksis and co-workers (8,9) separated the saturated and unsaturated triacylglycerols and examined the fatty acid composition of the classes thus separated. They observed that 52.6% of oleic acid in the sample was present as triacylglycerols containing two oleic acids. In other naturally occurring lipids, the more unsaturated fatty acid component is usually esterified in the 2-position of the glycerol moiety (26). Based on fatty acid data from human milk, one would expect to find increased linoleic and oleic acid concentrations in the 2-position and greater concentrations of saturated fatty acids in the 1- and 3-position. That such distribution is not predominant further emphasizes the distinct differences of human milk triacylglycerols from other triacylglycerols in nature.

As with adults, the fatty acid composition of infants' depot fat reflects the dietary fat intake (22). This was demonstrated by Widdowson *et al.* (27) in comparing the fat stores of English and Dutch infants. English infants, ingesting cow's milk formula, had approximately 3% linoleic acid in their subcutaneous fat; Dutch infants had approximately 35% linoleic acid in their fat stores as a result of the substitution of maize oil, high in linoleic acid, for the fat of cow's milk-based formulas. Gairdner (28) also noted that depot fat of breast milk-fed infants differed from that of formula-fed infants and reflected the fat composition of the diet.

Of particular importance to the neural and retinal development of the neonate is the presence in human milk of essential fatty acids and certain long-chain polyunsaturated fatty acids. Experiments in nonhuman primates have shown that n-3 fatty acids are necessary for normal

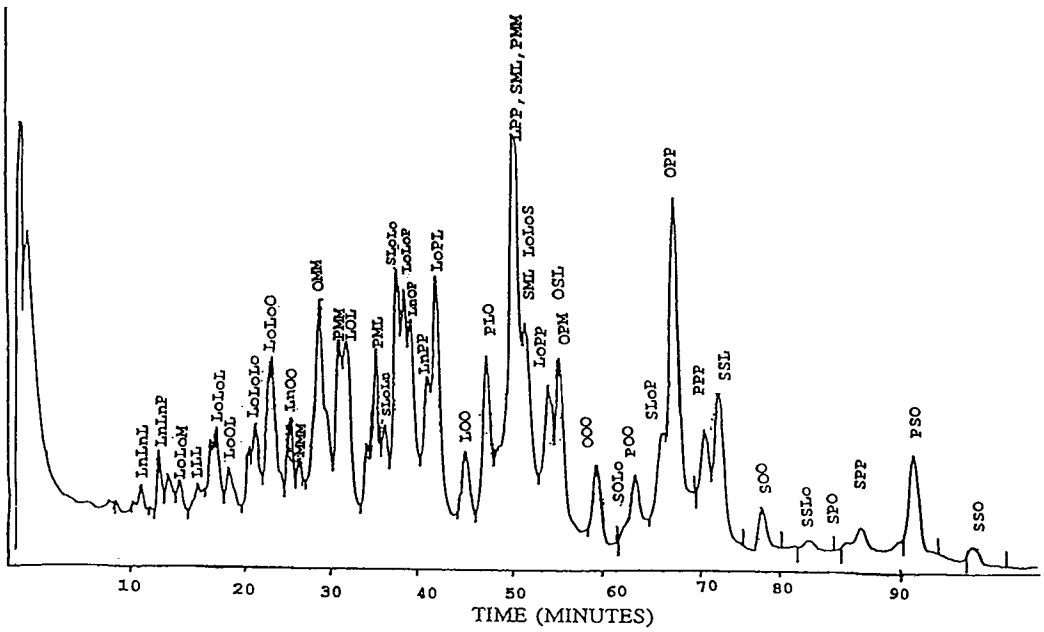

FIG. 4. Separation of human milk triacylglycerols by high-performance liquid chromatography using two 5-μ Supelcosil LC-18 silica columns in series with a mobile phase of acetone/acetonitrile (64:36, vol/vol).

TABLE 3

Human Milk Fatty Acid Composition[a]

Fatty acid	Weight %[b]	CV[c]
8:0	0.02	0.04
10:0	0.02	0.05
12:0	4.34	0.11
13:0	0.05	0.14
13:1n-1	0.03	0.10
14:0	4.65	0.07
14:1n-5	0.31	0.20
15:0	0.34	0.09
15:1n-5	0.09	0.09
16:0	19.25	0.03
16:1n-7	2.58	0.07
17:0	0.43	0.07
17:1n-7	0.33	0.07
18:0	7.97	0.06
18:1n-9	33.23	0.04
18:1 *t*	4.72	0.07
18:2n-6	15.55	0.05
18:3n-6	0.18	0.08
19:0	0.10	0.07
19:1n-9	0.11	0.08
18:3n-3	1.11	0.08
19:2n-6	0.51	0.05
20:0	0.21	0.10
20:1n-11	0.17	0.20
20:1n-9	0.38	0.11
20:2n-6	0.38	0.04
20:3n-6	0.46	0.05
20:4n-6	0.53	0.06
21:1n-9	0.01	0.29
20:3n-3	0.03	0.13
20:5n-3	0.07	0.12
22:0	0.12	0.06
22:1n-9	0.07	0.05
22:2n-6	0.05	0.06
23:0	0.03	0.02
22:L3n-3	0.13	0.05
23:1n-9	n.d.[d]	n.d.
22:4n-6	0.06	0.07
22:6n-3	0.16	0.08
24:0	0.22	0.14
24:1n-9	0.03	0.07
26:0	0.03	0.31

[a]Data represent weight percent of fatty acids identified *via* gas chromatography of fatty acid methyl esters using a Supelcowax 10 column (30 m × 0.25 mm i.d.) of 0.25 micron film thickness. An internal standard was used for quantitation.
[b]Mean of 30 samples.
[c]Coefficient of variation.
[d]Not detectable.

visual development (13,29,30). DHA appears to be of singular importance in both retinal and neural function. Human milk is the only infant food currently available which contains DHA (31); it also contains the important LC PUFA arachidonic acid (AA, 20:4n-6) and EPA. A physiological significance to the infant of DHA has not been demonstrated in humans. However, some recent studies (32-35) have shown an effect of differing n-3 fatty acid compositions in formula fed to premature infants; lowered erythrocyte DHA levels have been correlated with changes in electroretinogram, visual acuity and a test of infant memory. A specific requirement for DHA has not been demonstrated. Sinclair (36) showed that preformed dietary DHA was incorporated into the developing brain at ten times the rate of DHA formed from dietary linolenic acid by the infant, suggesting that the presence of DHA in human milk may confer a developmental advantage on breast-fed infants.

Although it is recognized that n-3 fatty acids are essential components of the diet, the Food and Nutrition Board of the National Research Council (37) has not established a neonatal requirement for n-3 fatty acids. Neuringer *et al.* (14) have suggested that the ratio of n-6/n-3 fatty acids present in human milk be used as a basis for dietary recommendations for normal neonatal development. This ratio appears to vary greatly among women and time periods, again reflecting the sensitivity of human milk fat to maternal dietary fat intake. The ratio reported for human milk is 4:1 to 10:1 (38,39). We have found a somewhat higher ratio in the milk of our subjects, all on self-selected Western diets; n-6/n-3 ratio averaged 12:1 and ranged from 2:1 to 22:1. Milk from our subjects contained approximately 18% of total fatty acids as n-6 fatty acids, in agreement with other data (4,6) from women on Western diets. We observed an average DHA concentration of 0.16 ± 0.01 weight %; Finley *et al.* (4) observed 0.23 ± 0.14% of DHA in milk of California women, while Bitman *et al.* (6) found a lower average of 0.06 ± 0.004% DHA in milk of Maryland women. The developmental significance of these levels of DHA in milk remains to be shown in human infants.

Using high-performance liquid chromatography and gas chromatography for dual analysis of the same samples, we were able to obtain quantitative measurement of triacylglycerols and fatty acids, including the n-3 and n-6 LC PUFA. We observed that the predominant triacylglycerols consisted of lauric, palmitic, stearic, oleic and linoleic acids. Four of the fatty acids, palmitic, stearic, oleic and linoleic acids, composed the major portion of the total fatty acids. Much variation in triacylglycerol and fatty acid composition was seen among subjects and time periods, reflecting dietary effects on milk lipid composition. Our data suggest that infants breast-fed by women on self-selected diets receive preformed LC PUFA from breast milk; however further analysis is necessary to understand the positional incorporation of these LC PUFA into milk triacylglycerols. The present method of triacylglycerol analysis is advantageous as a technique of sample analysis. Minor components may be concentrated from the sample for further analysis by collecting the triacylglycerol fractions as they elute from the column.

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COMMUNICATION

Fatty Acid Composition of Black Bear (*Ursus americanus*) Milk During and After the Period of Winter Dormancy[1]

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Black bears give birth and lactate during the 2-3-mon fast of winter dormancy. Thereafter the female emerges from the den with her cubs and begins to feed. We investigated fatty acid patterns of milk from native Pennsylvania black bears during the period of winter dormancy, as well as after den emergence. Throughout winter dormancy, milk fatty acid composition remained relatively constant. The principal fatty acids at all times were 14:0, 16:0, 16:1, 18:0, 18:1, 18:2n-6, 18:3n-3 and 20:4n-6. After den emergence, large changes occurred in almost all the fatty acids, particularly in 18:2n-6 and 18:3n-3. Large variability among the active free-ranging animals likely reflected differences in diet. In a carnivore, with apparently limited *de novo* synthesis of fatty acids, milk fatty acid composition may be affected by factors such as transition from reliance on stored lipids to feeding, and by temporal changes in dietary intake.
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The pattern of fatty acids in milk lipids varies considerably among mammalian species. Triglycerides (TG) account for 97-99% of milk lipid in the species that have been studied (1-3). However, among different species TG are composed to varying degrees of fatty acids originating from *de novo* synthesis within the mammary gland and/or from circulating fatty acids (4,5). The extent to which milk fatty acids reflect dietary or body storage lipids will depend on the extent of *de novo* synthesis. Other factors which may affect milk fatty acid composition include lactation stage and, in humans, gestation length (1,5-7).

Carnivores are believed to secrete milk lipids that result primarily from the uptake of circulating fatty acids and thus largely reflect dietary lipids. This is in contrast to ruminants, which secrete milk lipids high in short-chain fatty acids (4:0-6:0) of *de novo* origin, and some monogastrics (*e.g.*, rabbit, rat, mouse, elephant, and primates, including human), which secrete milk lipids high in medium-chain fatty acids (8:0-12:0) of *de novo* origin (4,5,8,9). The longer-chain fatty acids (≥ 16:0) may originate from either *de novo* synthesis or from uptake by the mammary gland. Although it is generally believed that the long-chain fatty acids are derived primarily from circulating blood lipids, the mammary gland of at least one species, the guinea pig, synthesizes primarily 16:0, as well as 18:0 and 18:1 (5,10,11). Unfortunately, fatty acid synthetase of the mammary gland has been studied in relatively few species, none of which are carnivores (5,12). Degree of plasma lipid uptake by the mammary gland also has been studied in only a few species, primarily in ruminants (9,12,13), and patterns may change with starvation (13).

Bears (family Ursidae) are of particular interest because they enter a period of winter dormancy, during which females give birth to and begin nursing their cubs (14). In the black bear *(Ursus americanus),* the first 2-3 mon of milk secretion occur while the female is fasting and mobilizing body lipid stores. Subsequently, the lactating female emerges from the den with her cubs and begins to feed on an omnivorous diet during the next months of lactation (15,16). Thus we would expect that milk fatty acids would reflect the composition of stored lipid in the denned-up period and dietary lipids thereafter. Although a number of studies on fatty acid composition of bear milks (17-20) have been undertaken, they have not been sufficiently detailed or comprehensive to permit an assessment of the effect of either winter dormancy or subsequent feeding on fatty acid composition.

The relationship of milk lipid composition to lactation stage is largely unknown for most free-ranging mammals. Prior reports on milk fatty acid composition for nondomestic species have usually been based upon analysis of only one or a few samples; information on lactation stage is often omitted, and it is sometimes unclear whether samples were taken from captive or wild individuals (*e.g.*, 18,21-23). In the present study we examined the fatty acid patterns of milk obtained from wild black bears at various stages of winter dormancy, as well as after den emergence. Our aims were to investigate i) whether changes occur in milk fatty acid composition during the prolonged fast in the den and ii) the extent to which milk fatty acid composition changes upon the transition from fasting to feeding.

MATERIALS AND METHODS

Milk samples were collected from a native population of black bears in the Poconos Mountains in eastern Pennsylvania. In Pennsylvania, as in other populations, females begin denning in late November and early December, about the same time that delayed implantation of the fertilized egg occurs (14-16). The average birth date is mid-January, and the female remains in the den with her cubs until late March or early April (15). Samples from the denned-up period were obtained from nine lactating females inhabiting natural or artificial dens in January [0-1 days *postpartum* (PP)], February (3-4 wk PP) and March (7-9 wk PP) 1984, corresponding to early (n=3), mid (n=3) and late (n=3) winter dormancy, respectively. Post-dormant samples (n=4) were collected from additional

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Abbreviations: ANOVA, analysis of variance; EFA, essential fatty acid; GC, gas chromatograph; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; PP, *postpartum*; PUFA, polyunsaturated fatty acid; TG, triglyceride.

free-ranging females from June to October (5–9 mon PP) of the same year. Females were immobilized with a mixture of ketamine HCl and xylazine delivered with a pole syringe (denned-up bears) or a dart (free-ranging bears). Milk was expressed manually following intramuscular injection of oxytocin. Milk samples were stored frozen at −20°C until analysis.

Total milk fat was determined gravimetrically using the standard Roese-Gottlieb procedure for milks (24). All solvents used for fatty acid analysis were high-performance liquid chromatography (HPLC) grade and all containers were composed of glass or teflon. Aliquots were extracted into chloroform using a modification (25) of the method of Folch *et al.* (26). Fatty acid methyl esters were prepared using the Hilditch reagent (0.5 N H_2SO_4 in methanol) (3) and extracted into isooctane for analysis. Methyl esters were separated by temperature-programmed gas-liquid chromatography (GLC) on a J&W DB-23 glass capillary column (30 m × 0.25 mm i.d.) (J&W, Folsom, CA) on a Shimadzu model GC-14A gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD). Carrier gas was helium. Starting column temperature was 153°C and temperature was increased stepwise to 220°C over a 30 min period. Peak identities were determined from known standards, and peak areas were quantified and normalized by automatic integration using a Shimadzu C-R4A Cromatopac. Fatty acids are herein designated by shorthand nomenclature of chain length:number of double bonds, where n-x denotes the position of the last double bond relative to the terminal methyl end. Data were tested by one-way analysis of variance (ANOVA) and subsequent Fisher PLSD multiple mean comparison tests or by two-tailed *t*-tests using a statistical package (Statview 512+, Brain Power Inc., Calabasas, CA) for the Macintosh. Data are presented as mean wt % ± SEM unless otherwise indicated.

RESULTS AND DISCUSSION

During the period of winter dormancy, fat content of the milk of eastern Pennsylvania black bears increases significantly as lactation progresses (27). In the present study, milk fat content was similar to that of the larger population, averaging 13.2 ± 2.75%, 22.8 ± 2.81% and 22.2 ± 3.09% at early, mid and late winter dormancy, respectively. However, despite the doubling of total fat content, milk fatty acid composition remained relatively constant throughout the period of dormancy (Fig. 1). The principal fatty acids of bear milk were 14:0, 16:0, 16:1n-9, 16:1n-7, 18:0, 18:1n-9, 18:1n-7, 18:2n-6, 18:3n-3 and 20:4n-6. During the winter dormancy, there was generally very little variability in levels of individual fatty acids among lactating females and little change occurred over time, except in levels of 16:0, 16:1n-9 and 20:4n-6, which decreased significantly after 1 day PP (ANOVA, Fig. 1). At all three stages, 16:0, 16:1, 18:0 and 18:1 accounted for about 85% of fatty acids. The major polyunsaturated (PUFA) and essential fatty acid (EFA) was 18:2n-6; the other principal PUFA were the EFA 18:3n-3 and 20:4n-6, although absolute levels were relatively low (Fig. 1). A number of minor fatty acids were also present at trace or very small amounts (≤ 0.6%) throughout lactation (Table 1).

Following the several months of lactation during winter dormancy, the female black bear emerges from the den

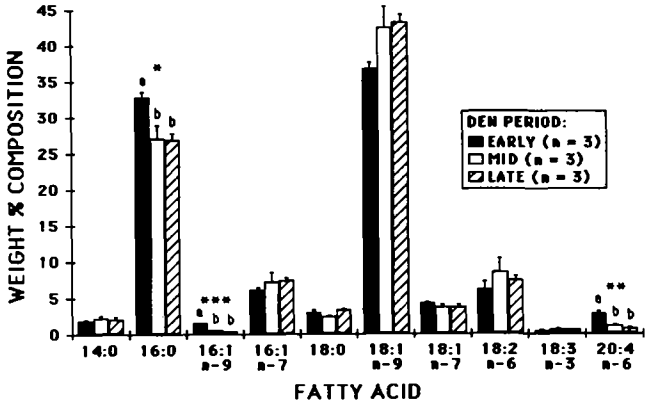

FIG. 1. Fatty acid composition of black bear milk over the period of winter dormancy. Early, 0–1 d *postpartum* (PP); mid, 3–4 wk PP; and late, 7–9 wk PP. Bars are means and vertical lines are 1 SEM. Means with different superscripts were significantly different (analysis of variance), *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

TABLE 1

Fatty Acid Composition of Black Bear Milk During and After the Period of Winter Dormancy[a]

Fatty acid	wt% Den (n=9)	wt% Post dormancy (n=4)
14:0	2.0 ± 0.16	3.2 ± 0.36[c]
16:0	28.9 ± 1.16	23.5 ± 1.74[b]
16:1n-9	0.8 ± 0.16	0.3 ± 0.04[e]
16:1n-7	6.9 ± 0.46	3.0 ± 0.38[d]
18:0	2.9 ± 0.21	5.8 ± 0.44[d]
18:1n-9	40.7 ± 1.42	37.2 ± 3.24[e]
18:1n-7	3.9 ± 0.17	1.6 ± 0.03[d]
18:2n-6	7.4 ± 0.74	15.7 ± 3.61[c]
18:3n-3	0.5 ± 0.06	5.1 ± 3.34[b]
20:4n-6	1.6 ± 0.34	0.3 ± 0.04[b]

[a] Values are means ± SEM. Minor components included 12:0 (0–0.1%), 14:1n-5 (0.2–0.3%), 17:0 (0.1–0.4%), 17:1 (0.3–0.6%), 18:1 (other isomers) (0.1–0.6%), 20:1 (0.4–0.5%), 22:1 (0.2–0.6%), 20:5n-3 (0–0.3%), 22:5n-3 (0–0.2%) and 22:6n-3 (0–0.2%). Data were tested by two-tailed *t*-test.

[b] $P < 0.05$, [c] $P < 0.01$, [d] $P < 0.001$, *t*-test.

[e] Not significant.

with her cubs and begins to roam in search of food (14,15). During this time, milk fat content was somewhat higher than it was earlier in lactation, averaging 28.0 ± 3.50%. Although there was little change or variability in milk fatty acid patterns over the period of winter dormancy, large changes occurred following emergence from the den (Table 1). Significant differences were found between the denned-up period and the active post-dormant period for every major fatty acid except 16:1n-9 and 18:1n-9. Levels of 14:0, 18:0 and 18:2n-6 approximately doubled after emergence, while levels of 16:1n-7, 18:1n-7 and 20:4n-6 were less than half those of the denned-up period. Most notable were the extremely high levels and considerable variability in the EFA 18:2n-6 and 18:3n-3 (Table 1). The levels of 18:2n-6, which averaged 7.4% during winter dormancy, were found to be as high as 21.6% after den

emergence. The levels of 18:3n-3 were even more remarkable. This fatty acid ranged between 0.2 and 0.8% during the denned-up period but was found to be as high as 15.0% during the active free-ranging period, with great variability among individual females. EFA intake may be particularly important to a bear cub because cubs are extremely small and altricial at birth [at <0.3% of maternal weight (14,15)], even for a carnivore (28). EFA appear to be adequately supplied in bear milk, with 18:2n-6 at about 6-13% of total calories over lactation, which is far in excess of human infant requirements (29,30).

In the present study, we found no evidence for *de novo* synthesis of short- or medium-chain fatty acids in the black bear; 12:0 was found in only trace amounts at a few stages (Table 1). Jenness *et al.* (31) came to the same conclusion with respect to a variety of bear milks, even though some reports from polar bears (17,19) and Yeso brown bears (20) indicate significant amounts of short-chain fatty acids. It is not clear whether the latter data may have been artifacts, as short-chain fatty acids are usually considered unique to ruminant milks (5). Whether the longer-chain fatty acids in bear milk derive from serum through uptake by the mammary gland or from *de novo* synthesis is not known. However, the long-chain n-6 and n-3 PUFA are all of dietary origin and, hence, must come from uptake.

During winter dormancy, milk lipids are secreted over an extended period of fasting. One might expect fatty acid composition to vary over this period due to changing rates of *de novo* synthesis, selective mobilization of depot fatty acids, or depletion of depot stores (*e.g.*, ref. 25), but the observed changes in fatty acid patterns were relatively minor (Fig. 1). Unfortunately, we were not able to compare milk fatty acid patterns to those of depot stores in these individuals. As such comparisons would need to be made in samples taken from the same animal (*e.g.*, ref. 25), data from other sources would not be appropriate.

The large changes that occurred after emergence (Table 1) presumably reflect dietary intake. Black bears feed primarily on a variety of fruits, nuts, acorns and succulent vegetation, as well as scavanged meat, human garbage, corn from farmed fields and, at least in Pennsylvania, handouts provided by human well-wishers (14-16). Hence the diets of individual bears may be highly variable. This may explain the high variability seen in the fatty acid patterns after emergence (Table 1). The high levels of 18:2n-6 and 18:3n-3 found in some plant materials, such as fruits, nuts, corn and leafy vegetation (*e.g.*, ref. 32), could account for the extraordinary levels found in some of the post-dormant milks. Lactating fur seals and sea lions that begin feeding after an initial fast also show substantial changes in milk fatty acid patterns with the onset of feeding (25,33). Significant increases in 18:2n-6 and 18:3n-3 have been observed in human milk lipids when subjects switch to vegetarian or corn oil-based diets (30). Although data is very limited, longer-chain PUFA have been reported in milks of the polar and grizzly bear (19). The diets of these animals are probably high in PUFA due to intake of fish and/or seal oils (*e.g.*, ref. 25).

Unfortunately, comparison of our data to those for other bears and other carnivores is difficult, given the limited nature of previous reports, as well as apparent misidentification of fatty acid peaks and/or failure to separate components with similar retention times. Species other than carnivores for which such very high levels of 18:3n-3 have been reported include the horse [n=1, (ref. 23)], koala [n=1, (ref. 23)] and red and mantled howler monkeys (wild, n=12, Iverson, S.J., and Oftedal, O.T., unpublished data), species which feed solely on vegetation.

Clearly, lactation stage and composition of the maternal diet are extremely important to the interpretation of milk fatty acid data in bears, and probably in other carnivores as well. In characterizing the fatty acid composition of any species, it is important to assay fatty acid composition on an adequate number of samples representing different lactation stages and, where known, maternal diet should be described. Results from this study also suggest that detailed data on milk fatty acid composition for free-ranging carnivores may allow inferences about the nature of their diet and thus may be useful in understanding both mammary gland physiology and the foraging ecology of a species, as has recently been demonstrated for several seal species (25,33).

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ARTICLE

The Existence of a Soluble Plasmalogenase in Guinea Pig Tissues

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The distribution of plasmalogenase for the hydrolysis of 1-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine) in the subcellular fractions of guinea pig tissues was examined. Plasmalogenase activity was found in high abundance in the cytosolic fractions of the brain and the heart. Assessment of microsomal marker enzyme activities in the cytosolic fraction revealed that plasmalogenase activity in the cytosol was not due to microsomal contaminations. The characteristics of the cytosolic plasmalogenase were very similar to the microsomal enzyme with respect to the pH profile of the reaction, the presence of divalent cations and K_m values for plasmenylethanolamine. However, the cytosolic enzyme was slightly less stable at 55°C than the microsomal enzyme. Cytosolic enzyme activity was eluted as a broad peak in Sepharose 6B chromatography with an average molecular weight of 250,000. Our results demonstrate that most of brain plasmalogenase activity is soluble which makes the brain cytosol an excellent source to initiate the purification of this enzyme.
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Phospholipids containing an *O*-alkenyl group at the C-1 position (plasmalogens) are abundant in many mammalian tissues (1). The most widely distributed plasmalogens are the 1-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine) species (1). Despite their ubiquitous distribution, only limited information is available on the metabolism or function of these phospholipids (2). The high concentrations of plasmenylethanolamines in electrically active tissues such as the brain and the heart imply that they may be involved in ion transport across membranes (3). Plasmalogens may also serve as reservoirs for prostaglandin precursors due to the large proportion of arachidonic acid at the C-2 position (4-6). Recently, a 1-alkenyl-2-acetyl-*sn*-glycero-3-phosphoethanolamine analogue of platelet activating factor has been identified in human neutrophils (7). The importance of plasmalogens to protect cell membranes from oxidative stress with the 1-alkenyl bond functioning as an oxygen radical scavenger has also been postulated (8).

The 1-alkenyl bond of plasmenylethanolamine can be hydrolyzed by two separate catabolic pathways. In the mammalian brain and heart, a microsomal plasmalogenase has been identified that cleaves plasmenylethanolamine to lysophosphatidylethanolamine and a fatty aldehyde (9,10). An alternate route for the cleavage of the vinyl ether bond of plasmenylethanolamine involves the action of a putative phospholipase A_2 followed by a lysoplasmalogenase. A lysoplasmalogenase activity has been characterized in both liver (11) and brain (12) microsomes. In view of the irreversible damage to both cerebral and cardiac tissues by oxidative stresses during and after ischemia (13) and the protective role plasmalogens play in this process (8), the identification and characterization of plasmalogenase activities in these tissues is highly desirable. In this study, the distribution of plasmalogenase activity in the subcellular fractions of the guinea pig tissues was investigated. Surprisingly, plasmalogenase activity was found in high abundance in the cytosolic fraction from guinea pig brain and heart. The characteristics of the cytosolic enzyme activity were studied and compared to the activity found in the microsomal fraction.

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Abbreviations: AU, absorbance unit; CDPethanolamine, cytidine diphosphoethanolamine; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. Aldehyde dehydrogenase, NAD^+, glutathione (reduced form), NADPH, cytochrome c (reduced form), iodine, potassium iodide, butylated hydroxytoluene, Triton QS-15, polyoxyethylene sorbitan monolaurate (Tween 20), and CDPethanolamine (cytidine diphosphoethanolamine) were purchased from Sigma Chemical Company (Toronto, Ontario, Canada). Thin-layer chromatography (TLC) plates (Sil-G25) were obtained from Fisher Scientific (Ottawa, Ontario, Canada). Silicic acid (BIO-SIL A) for column chromatography was a product of Bio-Rad Laboratories (Mississauga, Ontario, Canada). BDH Limited (Poole, England) provided the 2% dimethyldichlorosilane in 1,1,1-trichloroethane solution. Phospholipid standards were purchased from Serdary Research Laboratories (London, Ontario, Canada). Sepharose 6B for column chromatography was a product of Pharmacia LKB Biotechnology (Uppsala, Sweden). CDP[1,2-^{14}C]ethanolamine was a product of ICN Biomedicals (Costa Mesa, CA). All other chemicals were of the highest grade available and were acquired from the Canlab division of Baxter Diagnostics Corporation (Mississauga, Ontario, Canada). Guinea pigs weighing 275 ± 25 g were used throughout the study.

Preparation of plasmalogens for enzyme assays. Plasmalogens were prepared as previously described (10). Briefly, lipids were extracted from porcine hearts by the method of Folch *et al.* (14) in the presence of 0.5% butylated hydroxytoluene (wt/vol). The volume was reduced *in vacuo* and the lipid sample was dissolved in chloroform and applied to a silicic acid column. The individual phospholipids were eluted from the column with increasing amounts of methanol in chloroform (15). Fractions from the column were analyzed by TLC with a solvent containing $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (70:30:2:4, by vol). The fractions containing diradylglycerophosphoethanolamine were pooled. Phoshatidylethanolamine in the pooled diradylglycerophosphoethanolamine fraction was destroyed by the preferential hydrolysis of the ester bonds of phosphatidylethanolamine by mild alkaline hydrolysis with 0.35 M NaOH in 96% methanol as described by Renkonen (16). Lysoplasmenylethanolamine was prepared by hydrolyzing the diradylglycerophosphoethanolamine fraction in 0.35 M NaOH in 96% methanol for 45 min. The plasmenylethanolamine and lysoplasmenylethanolamine obtained after alkaline hydrolysis were repurified by

silicic acid column chromatography. The purity of the plasmalogen fractions was assessed by determining the ratio of the vinyl ether content and the total phosphorus content in the samples. Only preparations with purity greater than 96% were used for enzyme assays.

Preparation of subcellular fractions from guinea pig tissues. Guinea pig tissues were removed and placed on ice. The tissue was homogenized in 0.25 M sucrose/10 mM Tris-HCl (pH 7.4) with two 20 s bursts of a Polytron probe (PT-30) (Brinkmann Instruments; Rexdale, Ontario, Canada) at a speed setting of six. Alternatively, the brain tissues was homogenized in a Potter-Elvehjem homogenizer equipped with a teflon pestle. The tissue homogenates were centrifuged at 10,000 × *g* for 20 min and the supernatant was centrifuged again at 100,000 × *g* for 60 min. The supernatant obtained from the last centrifugation was removed with a Pasteur pipette and designated as the cytosolic fraction. The precipitate containing the microsomal pellet was dispersed in the homogenizing buffer with a Dounce homogenizer equipped with a type A pestle.

Plasmalogenase assay. All glassware including test tubes were treated with a 2% dimethyldichlorosilane in 1,1,1-trichloroethane solution to minimize the adherence of lipids to glass containers. Plasmalogenase activity was monitored by the disappearance of the substrate (9,10). Purified plasmenylethanolamine (2 μmol) was suspended in 1 mL of 10 mM Tris-HCl (pH 7.4) containing 0.05% Tween 20. The mixture was sonicated in a water bath until translucent. The reaction mixture (1.5 mL) contained 300 nmol dispersed plasmalogen, 50 mM Tris-HCl (pH 7.4) and an enzyme preparation containing 0.75–1.0 mg of protein. The reaction was initiated by the addition of the enzyme, and the mixture was incubated at 37°C for 15 min. Control tubes contained either no enzyme or enzyme that had been incubated at 100°C for 5 min. At 0 and 15 min of incubation, 600 μL was removed from the reaction mixture and placed into a tube containing 1.5 mL $CHCl_3$/CH_3OH (2:1, vol/vol). Water and chloroform were added to cause phase separation. The upper phase was removed, and an aliquot of the lower phase was assayed for vinyl ether content spectrophotometrically at 355 nm. Plasmalogenase activity was calculated from the difference in vinyl ether content between the 0 and 15 min time points. Total enzyme activity was calculated from the product of the specific activity of the enzyme and the total protein content in the subcellular fraction.

The determination of marker enzyme activities. The degree of microsomal contamination in the cytosolic fraction was determined by the activities of known microsomal marker enzymes in the cytosol. NADPH-cytochrome c reductase (17), phosphoethanolaminetransferase activities (18) and 5′-nucleotidase activities (19) were used as microsomal markers.

Analytical procedures. Lipid phosphorus was assayed by the method of Bartlett (20). Plasmalogen (vinyl ether) content in the sample was assessed by the method of Gottfried and Rapport (21) based on iodine determination. The correction factor for alkylacyl compounds was not used for the calculation of vinyl ether content. Protein was determined by the method of Lowry *et al.* (22). All results are expressed as the mean ± standard deviation of at least three separate experiments except where otherwise indicated. The points on all figures have standard deviations of less than 15% of the mean.

RESULTS AND DISCUSSION

Plasmalogenase assays. The disappearance of plasmenylethanolamine was monitored by the loss of the vinyl ether bond. A typical assay resulted in the disappearance of 10–20 nmol of the vinyl ether bond of plasmenylethanolamine over the 15 min incubation period. This change in vinyl ether content resulted in an increase in absorbance of 0.040–0.080 A.U. at 355 nm (Fig. 1) which could be easily detected by a modern spectrophotometer. Enzyme activity was linear with protein concentration between 0.5–3.0 mg of protein from guinea pig brain cytosol or microsomes.

Subcellular localization of plasmalogenase activity. The distribution of plasmalogenase activities in the subcellular fractions of guinea pig brain, heart and liver was investigated. The enzyme activity was assessed by the disappearance of the substrate during the reaction. A small amount of plasmalogenase activity was found in the mitochondrial fraction of the brain and heart (data not shown); however, most of the enzyme activity (>90%) was located in the microsomal and cytosolic fractions. As depicted in Table 1, 83% of the total brain enzyme activity was found in the cytosolic fraction, whereas 68% of the total heart enzyme was located in the cytosol. A similar distribution of plasmalogenase activity was also observed in the subcellular fractions of the rat brain and heart (data not shown). The distribution of enzyme activity between the cytosol and microsomes was not affected by the mode of tissue homogenization. Plasmalogenase activity in the liver had a similar subcellular distribution, but the activity was very low.

In order to determine if the hydrolysis of the vinyl ether group of the plasmenylethanolamine by guinea pig brain cytosol was due to the direct acton of the plasmalogenase or the combined action of phospholipase A_2/lysoplasmalogenase, the reaction mixture was analyzed for the disappearance of plasmenylethanolamine as well as the formation of the radylglycerophosphoethanolamine. The reaction mixture contained 300 nmol of plasmenylethanolamine, and the assay for plasmalogenase activity was carried out as described in the preceding section. The reaction was terminated by the addition of 3 mL of $CHCl_3$/CH_3OH (2:1, vol/vol), and the lipids in the lower phase

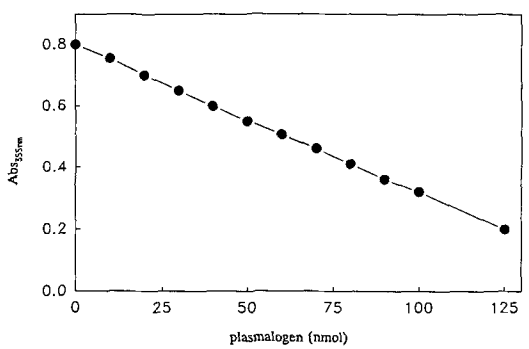

FIG. 1. The relationship of plasmenylethanolamine content *vs.* absorbance by the determination of the vinyl ether group. Each point is the mean of four separate experiments. An identical curve was obtained when lysoplasmenylethanolamine was used as the vinyl ether source.

TABLE 1

Plasmalogenase Activities in the Subcellular Fractions of Guinea Pig Tissues[a]

Tissue	Specific activity: Plasmenylethanolamine disappearance (nmol/h/mg protein)	Total activity[b]: Distribution (%)
Brain		
Cytosol[c]	89.8 ± 7.3	83%
Microsomes[c]	66.1 ± 3.6	17%
Cytosol[d]	84.2 ± 7.9	78%
Microsomes[d]	77.8 ± 13.1	22%
Heart		
Cytosol	47.6 ± 5.8	68%
Microsomes	57.0 ± 8.1	32%
Liver		
Cytosol	6.5 ± 2.5	75%
Microsomes	5.1 ± 1.5	25%

[a] Plasmalogenase activities were determined by substrate disappearance as described in Materials and Methods. Each value represents the mean ± standard deviation of at least four different experiments.

[b] Total activity was calculated from the specific activity of the enzyme and the amount of protein in each subcellular fractions.

[c] Prepared by homogenization with a Polytron generator equipped with a PT-30 probe.

[d] Prepared by homogenization with a Potter-Elvehjem homogenizer equipped with a teflon pestle.

were separated by TLC with a solvent containing $CHCl_3$/CH_3OH/CH_3COOH/H_2O (70:30:2:4, by vol). At the 0 min time point (control), no radylglycerophosphoethanolamine fraction was detected after TLC separation. After 15 min of incubation, 22 ± 3 nmol of plasmenylethanolamine was hydrolyzed with the formation of 14 ± 2 nmol of radylglycerophosphoethanolamine. Analysis of the vinyl ether content in the radylglycerophosphoethanolamine fracton revealed that there was no detectable vinyl ether group in this fraction. Our results indicate that the majority of the plasmenylethanolamine was hydrolyzed by plasmalogenase and not by a phospholipase A_2/lysoplasmalogenase system.

To further confirm the above results, time courses for the hydrolysis of plasmenylethanolamine and lysoplasmenylethanolamine in guinea pig brain cytosol were determined (Fig. 2). The hydrolysis of plasmenylethanolamine was linear up to 30 min. However, the brain cytosol had very limited ability to hydrolyze lysoplasmenylethanolamine. The results obtained in this study support the notion that the hydrolysis of plasmenylethanolamine resulted from the direct action of plasmalogenase in the brain cytosol.

It could be argued that the plasmalogenase activity in the brain and heart cytosol might arise from the contamination of microsomal particles. Hence, the microsomal marker enzyme activities in the cytosolic fractions were assessed (Table 2). The result shows that the contamination of the cytosolic fraction by microsomal enzyme markers did not exceed 12% in any of the cases. Hence, the plasmalogenase activities in the cytosolic fraction of the brain and heart could not arise solely from microsomal contamination.

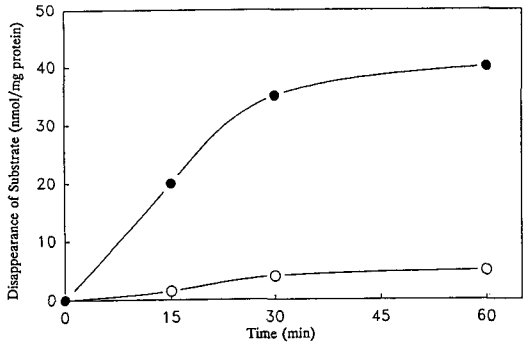

FIG. 2. Time course for the hydrolysis of plasmenylethanolamine and lysoplasmenylethanolamine in guinea pig brain cytosol. Enzyme activities (1.25 mg cytosolic protein) were assayed at 37°C in the presence of 300 nmol plasmenylethanolamine (●) or lysoplasmenylethanolamine (○) by measuring substrate disappearance as described in Materials and Methods. Each point represents the mean of three separate experiments.

TABLE 2

The Distribution of Microsomal Marker Enzyme Activities in the Microsomal and Cytosolic Fractions of Guinea Pig Brain and Heart

Enzyme		Percentage distribution[a]: Brain	Heart
NADPH-cytochrome c reductase	Cytosol	12	—
	Microsomes	88	—
5′-Nucleotidase	Cytosol	—	2
	Microsomes	—	98
Phosphoethanolamine transferase	Cytosol	6	N.D.[b]
	Microsomes	94	100

[a] Each value is the average of three separate experiments.

[b] N.D., not detectable.

Characterization of plasmalogenase activities. In view of the abundance of soluble plasmalogenase in the brain, its cytosol was employed for further characterization of the enzyme activity. The effect of substrate concentrations on plasmalogenase activity was investigated (Fig. 3). From the double reciprocal plot of enzyme activity *vs.* plasmenylethanolamine concentrations, the K_m of the enzyme for plasmenylethanolamine was estimated to be 154 μM. The K_m of plasmenylethanolamine for the cytosolic enzyme is comparable to the microsomal enzyme (105 μM) and also the enzyme obtained from an acetone extract of the bovine brain (285 μM) (9). Similar to the microsomal enzyme (data not shown), the cytosolic enzyme from the brain was also inhibited by 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM Mn^{2+}. However, the presence of 1 mM Ca^{2+} or Mg^{2+} had very little effect on either enzyme activity (Table 3) (10). Taken together, our results show that plasmalogenase activity in the cytosol may require a minimum level of metalic cation(s) for full activity. The pH profiles of the brain cytosolic and microsomal plasmalogenase activities were determined (Fig. 4). Both enzymes displayed similar pH profiles with optima at pH 7.5. When the enzymes from

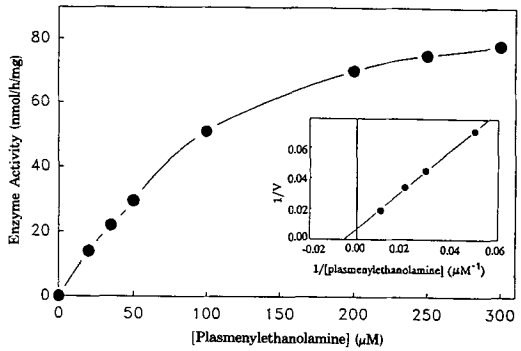

FIG. 3. Effect of substrate concentrations on guinea pig brain cytosolic plasmalogenase activity. Plasmenylethanolamine (2 μmol) was suspended in 1 mL of 10 mM Tris-HCl/0.05% Tween 20 by sonication and appropriate aliquots of the suspension were added to the enzyme assays. Each assay contained 1.25 mg of cytosolic protein and the reaction was carried out at 37°C for 15 min as described in Materials and Methods. Inset is the Lineweaver-Burke plot of the same data. Each point represents the mean of three separate determinations.

TABLE 3

Effects of EDTA and Cations on Cytosolic Plasmalogenase Activity of Guinea Pig Brain[a]

Cation (1 mM)	Enzyme activity (% control)
Control	100
Ca^{2+}	85
Mg^{2+}	100
Mn^{2+}	N.D.[b]
EDTA	N.D.[b]

[a] Plasmalogenase activities were assayed by the substrate disappearance method as described in Materials and Methods, in the absence (control) or presence of 1 mM of various cations or ethylenediaminetetraacetic acid (EDTA). Each value is the average of three separate experiments.

[b] N.D., not detectable.

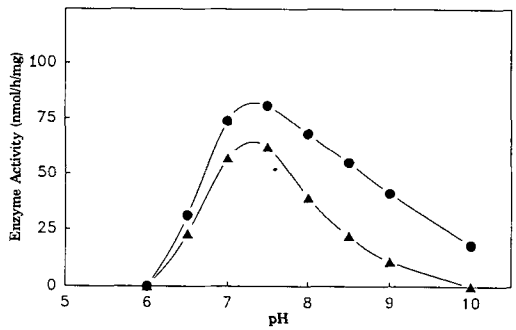

FIG. 4. The pH profile of guinea pig brain cytosolic and microsomal plasmalogenase activities. Enzyme activities in the cytosolic (●) and microsomal (▲) fractions were assayed by substrate disappearance as described in Materials and Methods section. Each assay contained 1.25 mg cytosolic protein or 0.8 mg microsomal protein and the reactions were incubated at 37°C for 15 min. Tris-succinate buffer was used from pH 6–7, and Tris-HCl was used from pH 7–10. No significant difference was detected in enzyme activity at pH 7 when either buffer was utilized. Each point represents the mean of four separate experiments.

the microsomal and cytosolic fractions were incubated at 50°C and 55°C (Fig. 5), the microsomal enzyme was more stable than the cytosolic form.

Gel filtration chromatography of guinea pig brain cytosol. Gel filtration chromatography was employed to confirm the true solubility of the brain cytosolic plasmalogenase. The brain cytosol (2 mL) was applied to a Sepharose 6B column (3 × 48 cm) equilibrated with 0.1 M Tris-HCl (pH 7.5). Subsequent to sample application, the column was washed with the same buffer, and fractions of 1.2 mL were collected and assayed for enzyme activity (Fig. 6). Plasmalogenase activity was eluted as a broad peak away from the void volume of the column. The fraction with the highest enzyme activity had an apparent molecular weight of 250,000. The broad and asymmetrical

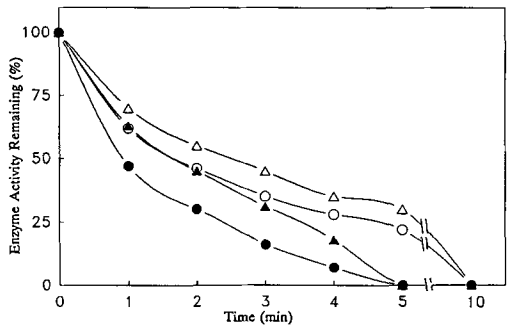

FIG. 5. Effect of heat treatment on plasmalogenase activities in the cytosol and microsomes of the guinea pig brain. Aliquots of brain cytosol (○, ●) and microsomes (△,▲) were incubated at 50°C (open symbols) or 55°C (closed symbols) for 0–5 min. Each aliquot contained 1.25 mg cytosolic protein or 0.8 mg microsomal protein. Subsequent to incubation, enzyme activities in the aliquots were determined by the disappearance of substrate as outlined in Materials and Methods. Each point is the mean of three separate experiments.

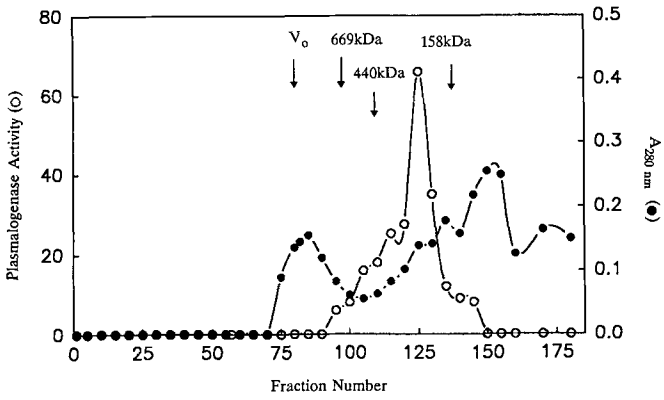

FIG. 6. Sepharose 6B chromatography of plasmalogenase activity in guinea pig brain cytosol. Guinea pig brain cytosol (2 mL) containing 16 mg of protein was applied to a Sepharose 6B column (3.0 × 48 cm) equilibrated with 100 mM Tris-HCl (pH 7.5). Subsequent to sample application, the column was washed with the same buffer and 1.2 mL fractions were collected. An aliquot (0.8 mL) of the fraction was used for enzyme activity determination and the activity is expressed as nmol/h/mL. The total yield of plasmalogenase activity after column chromatography was 41 ± 5% of the enzyme activity applied to the column. The void volume of the column is indicated by V_o. Thyroglobulin (669 kDa), ferritin (440 kDa) and aldolase (158 kDa) were used for column calibration.

peak of enzyme activity implies that the enzyme was eluted as multimeric proteins or in aggregation with other cytosolic proteins. An alternate explanation is that the enzyme might still be associated with some lipid molecules present in the cytosol. The fact that none of the enzyme activity was eluted near the void volume of the column suggests that it was not complexed with microsomal or large liposomal particles.

In a previous study, the existence of a metabolite in the rat brain cytosol for the elimination of the vinyl ether bond of plasmenylethanolamine was reported (23). The material was thermostable at 100°C, had a low molecular weight and was found to be a non-protein entity which was later identified as ascorbic acid (24). Hence, our study is the first identification of a truly soluble plasmalogenase from mammalian sources. In the last two decades, the existence of plasmalogenase activity in the brain has been a matter of debate. Plasmalogenase activity was identified in the microsomes of rat brain (9), in neuronal perikarya, astroglia, and oligodendroglia from bovine brain (25), and from the brains of rats and monkeys (26). Using another approach, it was shown that the rat brain had no ability to catabolize radiolabeled plasmalogens, but low levels of lysoplasmalogenase activity were detected in brain (12). Our results support the existence of considerable amounts of plasmalogenase activity in the brain and also the presence of low levels of lysoplasmalogenase activity. At present, the reason for the discrepancy in the identification of plasmalogenase activity is not entirely clear. One explanation is that the activity of the plasmalogenase is highly dependent on the source of the plasmalogen and the method of suspension in the buffer. The assay procedure in this study had been optimized to provide a high plasmalogenase activity.

The similarity in characteristics between the cytosolic and microsomal enzyme makes it plausible to speculate that both enzymes may originate from the same protein. However, the ability to obtain the same distribution of enzyme activity between the two compartments by different methods of homogenization confirms that the cytosolic enzyme was not mechanically detached from the microsomes during tissue homogenization. The identification of a truly soluble plasmalogenase makes the guinea pig brain cytosol an ideal source of the enzyme for its subsequent purification. At present, the physiological significance of the distribution of the enzyme in two subcellular compartments remains undefined.

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Adipose Hormone-Sensitive Lipase Preferentially Releases Polyunsaturated Fatty Acids from Triglycerides

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Rat adipose hormone-sensitive lipase-mediated release of fatty acids from triglycerides was studied in three model systems: i) cultured preadipocytes containing polyunsaturated fatty acid-enriched triglyceride; ii) perfused epididymal fat pads; and iii) *in vitro* incubations of crude preparations of hormone-sensitive lipase with synthetic triglyceride-analogues as substrates. We found that cultured preadipocytes challenged with 10 μM norepinephrine tended to release more ω6 and ω3 polyunsaturated fatty acids than saturated fatty acids. Fat pads perfused with 10 μM norepinephrine preferentially released arachidonate and α-linolenate but tended to retain oleate and linoleate. Finally, crude preparations of hormone-sensitive lipase released from the triglyceride-analogue substrates α-linolenate twice as fast as oleate. We conclude that rat adipose hormone-sensitive lipase preferentially releases polyunsaturated fatty acids from triglycerides. We suggest that this may be a mechanism by which these fatty acids are kept from being trapped in fat depots and maintained in the circulation.
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The control of the flux of polyunsaturated fatty acids (PUFA) in adipose tissue is not completely understood. While it is true that adipose fatty acid profiles correlate with dietary lipid composition (1), the ratio of PUFA to saturated fatty acids as well as the ratio of ω3 to ω6 PUFA in adipose tissue is consistently lower than that in the diet (2–7). Furthermore, adipose tissue contains a higher proportion of saturated fatty acids over PUFA relative to other tissues and organs in the body (2,3,8–14). The tendency to store saturated fatty acids rather than PUFA ensures that PUFA precursors of eicosanoids are not trapped within adipose tissue.

We have investigated the ability of hormone-sensitive lipase (HSL) to release PUFA from triglycerides (TG) as a factor in maintaining a low relative content of PUFA in adipose tissue. We have approached this problem using three models: cultured rat perirenal preadipocytes, perfused epididymal fat pads, and a pH 5.2 precipitate preparation of adipose hormone-sensitive lipase.

EXPERIMENTAL PROCEDURES

Cell culture and fatty acid release. We have previously established the culture conditions necessary to enrich differentiating cultured preadipocytes with PUFA (15). Briefly, the culture medium of differentiating preadipocytes undergoing TG-loading was supplemented with 50μM fatty acid (NuChek Prep, Elysian, MN) complexed to CPSR-1 serum replacer (Sigma, St. Louis, MO). Fat-loading of the cells was monitored by gas-chromatographic quantification of intracellular TG (15) and by the accumulation of visually-apparent intracellular lipid droplets in cultures stained with osmium tetroxide (16). TG-loaded cells were rinsed twice with M199E (Sigma) containing 10 mM glucose, penicillin (100 U/mL), streptomycin (0.1 mg/mL) and 2% (wt/vol) fatty acid-free bovine serum albumin (Sigma) to ensure complete removal of treatment fatty acids and insulin. The rinsed cells were incubated in the same medium used to rinse the cells, plus 10 μM norepinephrine (Boehringer, Montréal, Québec Canada) for 24 h. The medium was changed once at 6 h during this 24-h incubation with norepinephrine. Intracellular TG before and after treatment with norepinephrine was analyzed by gas chromatography as described previously (15). The relative content of PUFA of the cells is expressed as the ratio of total PUFA, ω6 PUFA or ω3 PUFA to total saturated fatty acids, symbolized as P/S, P(ω6)/S and P(ω3)/S, respectively.

Epididymal fat pad perfusion. The technique for surgery, cannulation and perfusion of the epididymal fat pad was described by Ho and Meng (17) and followed without modification. The isolated fat pad was perfused in open circuit at 37°C with oxygenated Krebs-Ringer buffer containing 10 mM glucose and 4% (wt/vol) fatty acid-free bovine serum albumin at 50 μL/min × g tissue. After an initial 30-min perfusion period, 10 μM norepinephrine was added to the influent. The effluent during the norepinephrine infusion was collected and its non-esterified fatty acid (NEFA) profile analyzed by gas chromatography and compared with the TG-fatty acid profile of the fat pad.

Synthesis of [^{3}H]oleoyl-[^{14}C]linolenoyl-2-O-(9-octadecenyl)glycerol as substrate for HSL. Ten μmol each of [^{3}H]oleic acid and [^{14}C]α-linolenic acid (specific activities of 4μCi/μmol) were mixed and converted to acyl chlorides using oxalyl chloride as described previously (18). To the fatty acyl chlorides was added 1.5 mL of toluene containing 6 μmol 2-*O*-(9-octadecenyl)glycerol (Serdary Research Laboratories, London, Ontario, Canada) and 50 μM 4-pyrrolidinopyridine (Sigma). The mixture was stirred overnight at 50°C (19), and the product was extracted with diethyl ether/hexane (1:1, vol/vol). The organic layer containing the reaction products was washed with 2 M HCl followed by water. The washed organic layer was evaporated to dryness, and the residue purified by thin-layer chromatography (TLC) using hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol) as developing solvent. The band corresponding to diacyl-2-*O*-alkylglycerol was further fractionated by argentation TLC to separate the desired product, *rac*[^{3}H]oleoyl-[^{14}C]linolenoyl-2-*O*-(9-octadecenyl)-glycerol, from the dioleoyl and dilinolenoyl derivatives of 2-*O*-(9-octadecenyl)glycerol. The $AgNO_3$/TLC plates were prepared by dipping pre-coated Whatman LK6D silica gel plates (Chromatographic Specialties, Brockville, Ontario, Canada) into a 4% (wt/vol) $AgNO_3$/15% (vol/vol) NH_3/1% (vol/vol) methanol solution and activating them at 200°C for 2 h. Samples were applied and the $AgNO_3$ plates were developed twice in diethyl ether/benzene (2:8, vol/vol).

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Abbreviations: HSL, hormone-sensitive lipase; NEFA, non-esterified fatty acids; PUFA, polyunsaturated fatty acids; TG, triglycerides; TLC, thin-layer chromatography.

Fractions were visualized with 1% dichlorofluorescein, and the band corresponding to 5 double bonds was scraped and eluted as described (20,21). The purified product was analyzed by gas chromatography using triheptadecanoylglycerol as internal standard, and sodium methoxide as the transmethylating agent. The fatty acid methyl esters obtained were separated from the parent 2-*O*-(9-octadecenyl)glycerol by TLC prior to injection into the gas chromatograph. The final product was 99% pure and contained equimolar quantities of esterified oleic and α-linolenic acids. The overall yield was 5–10%.

Preparation and assay of crude HSL. Sprague-Dawley male rats (200–225 g, Charles River, St. Constant, Québec Canada) were starved overnight and sacrificed by decapitation. Epididymal and perirenal fat pads were immediately excised and processed as described previously (22) up to the "pH 5.2 precipitate fraction" containing the HSL activity. The activity of protein kinase-activated HSL was determined using glycerol-dispersed solutions of substrates (23). The release of NEFA was measured by direct extraction (24) or by TLC (25).

RESULTS

We have previously shown that cultured differentiating perirenal preadipocytes when undergoing fat-loading also accumulate significant proportions of PUFA in TG when the lipid-poor culture medium is enriched with PUFA (15). Using this technique, we prepared TG-loaded control, oleate-, linoleate-, α-linolenate-, arachidonate- and docosahexaenoate-enriched perirenal preadipocytes. These cells released NEFA and lost intracellular TG when the insulin and serum supplement in the culture medium were replaced with 10 μM norepinephrine and 4% fatty acid-free bovine serum albumin (Fig. 1). After a 24-h treatment with norepinephrine, the fat-loaded cells lost an average of 1680 nmol/9.6 cm^2 flask of TG-esterified fatty acid ($P < 0.001$, two-way analysis of variance, pooled SEM = 330 μmol, n = 36). The decrements in intracellular TG expressed as μmol fatty acid/9.6 cm^2 flask and in percentage of the initial TG (given in parenthesis) for each group were: control, 2300 (70%); oleate-enriched, 1100 (40%); linoleate-enriched, 2700 (66%); α-linolenate-enriched, 600 (27%); arachidonate-enriched, 1600 (53%); and docosahexanoate-enriched, 1500 (70%). The cells lost an average of 54% ± 10 intracellular TG. Norepinephrine did not affect the total phospholipid content of the cells (data not shown).

The norepinephrine-induced hydrolysis of TG shows a significant degree of specificity for PUFA (Fig. 2A). After the 24-h incubation with norepinephrine, the P/S ratios of intracellular TG dropped by an average of 33% (28 to 44%) in preadipocytes enriched with linoleate, α-linolenate, arachidonate and docosahexaenoate. Norepinephrine had no effect on the P/S ratios in oleate-enriched cells. The apparent drop in the P/S ratio in the linoleate group was not statistically significant. Calculation of the proportion of ω6 PUFA relative to saturated fatty acids in preadipocyte intracellular TG (Fig. 2B) showed an average drop of 33% (26 to 42%) in the P(ω6)/S ratio as a result of norepinephrine treatment. Similarly, the proportion of ω3 PUFA in preadipocyte TG (Fig. 2C) also dropped by 30% and 36% for α-linolenate- and docosahexaenoate-enriched cells,

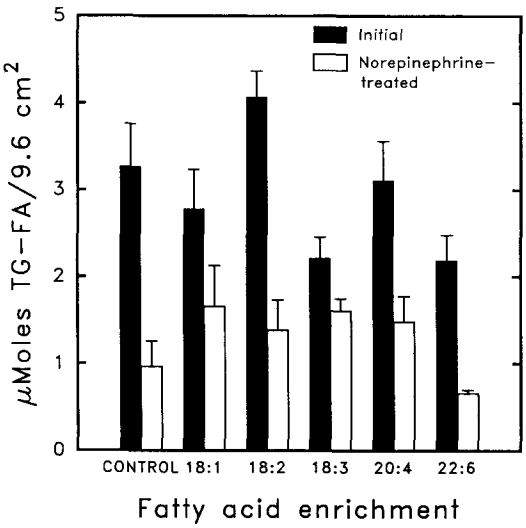

FIG. 1. Norepinephrine-induced loss of intracellular triglycerides (TG) from cultured perirenal preadipocytes. TG of differentiating cells were enriched with the indicated fatty acids, after which the cells were treated with 10 μM norepinephrine for 24 h. Intracellular TG was measured and compared with that of non-treated cells. Values represent the mean ± SEM of three experiments.

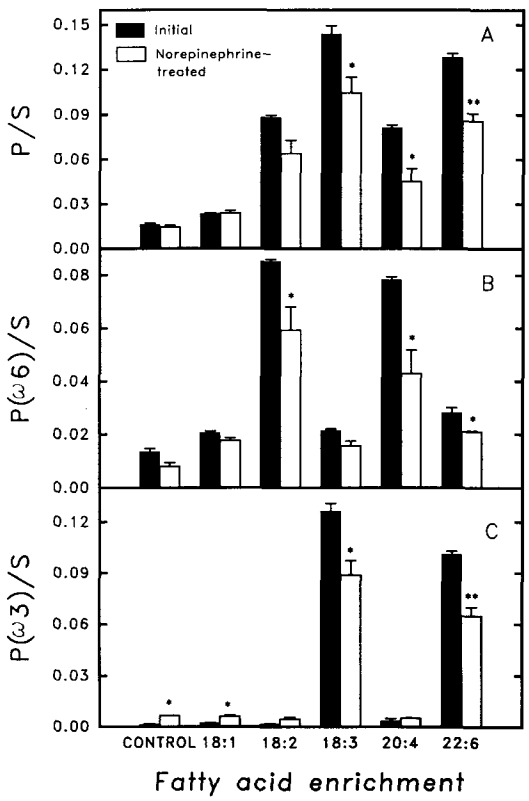

FIG. 2. Preferential release of polyunsaturated fatty acids (PUFA) from pre-adipocytes. Triglycerides (TG) of cells were enriched with the indicated fatty acids; then the cells were treated with 10 μM norepinephrine. The fatty acid composition of intracellular TG was determined and compared with that of non-treated cells. The results, mean ± SEM of three experiments, are expressed as: A) P/S ratios; B) ω6 PUFA to S (P(ω6)/S) ratios; and C) ω3 PUFA to S (P(ω3)/S) ratios. Statistical significance is indicated by a "*," $P < 0.05$, or by "," $P < 0.01$ (two-tailed Student's *t*-test).**

respectively. However, there was a statistically significant 2.4-fold and 2.1-fold enrichment in ω3 PUFA of TG in control and oleate-enriched cells, respectively (Fig. 2C). This suggests conservation of ω3 PUFA in cells with a very low content (<1%) of ω3 PUFA.

The data show that the drop in the overall P/S ratio is accounted for by the preferential release of both ω6 and ω3 PUFA from preadipocyte TG. The preferential retention of oleate in intracellular TG (Fig. 3) upon norepinephrine-stimulation of TG hydrolysis indicates that the preferential release of PUFA cannot be adequately explained by the "last-in first-out" hypothesis for fatty acids widely accepted for adipose tissue (26–28). Results from 6-h treatment of cells with norepinephrine gave similar trends, with an average release of 10% of intracellular TG-esterified fatty acid (results not shown).

The results from the epididymal fat pad perfusion (Fig. 4) support the hypothesis that adipose preferentially releases PUFA over saturated fatty acids. Norepinephrine increased the rate of release of glycerol into the effluent from a basal rate of 0.3 μmol/h to 1.1 μmol/h (single experiment), indicating stimulation of TG hydrolysis in the perfused fat pad. The data shown in Figure 4 represent ratios between the relative percent of selected fatty acids exiting with the effluent from the fat pad, and the relative percent of the corresponding fatty acid in the fat pad after the perfusion experiment. Except for palmitate, the effluent/tissue fatty acid ratios were significantly different from unity ($P < 0.05$, two-tailed Student's t-test). Values greater than unity represent preferential release of the fatty acid. Figure 4 shows that there is a 3-fold greater concentration of arachidonate in the effluent than in the TG of the perfused fat pad indicating that this fatty acid is preferentially released. Consistent with the preadipocyte data, the fat pads tend to preferentially release α-linolenate and to preferentially retain oleate. In contrast to the preadipocyte data, the perfused fat pads tend to retain linoleate. It is unlikely that PUFA enrichment of the effluent was the result of phospholipase C activation by α_1-receptor activity. Brooks *et al.* (29) had shown that

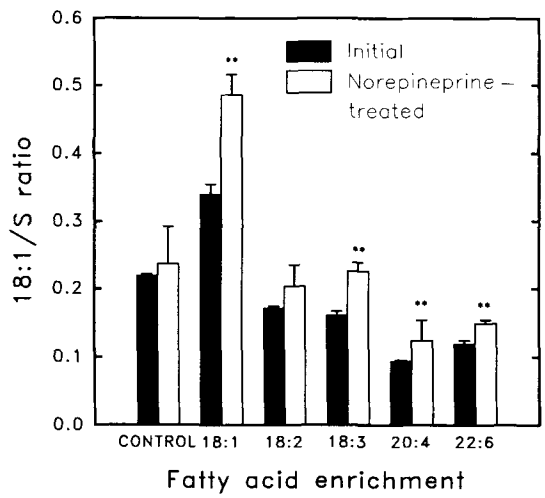

FIG. 3. Preferential retention of 18:1 in pre-adipocyte triglycerides (TG). Cells were treated and intracellular TG analyzed as in Figures 1 and 2. The results, mean ± SEM of three experiments, are expressed as 18:1/S ratios. Statistical significance is indicated by "," $P < 0.01$ (two-tailed Student's t-test).**

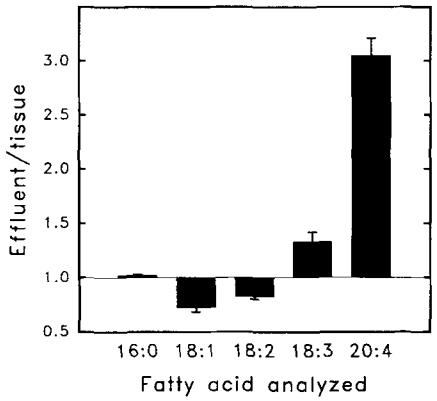

FIG. 4. Preferential release of 18:3 and 20:4 from norepinephrine-stimulated, perfused epididymal fat pads. The fat pads were perfused for one hour at 37°C with Krebs-Ringer bicarbonate buffer with 10 mM glucose, 4% fatty acid-free bovine serum albumin and 10 μM norepinephrine. The fatty acid composition of the effluent nonesterified fatty acids and of fat pad TG were analyzed. The results, expressed as mean ± SEM of three perfusions, are given as the ratios of the relative proportions of the indicated fatty acids.

norepinephrine-induced fatty acid release by adipocytes obtained from rat epididymal fat pads is due to β-receptor activity. Furthermore, phospholipase C acts primarily on phosphoinositides (30), an extremely small pool relative to intracellular TG in adipocytes.

The crude preparations of epididymal and perirenal HSL, the "pH 5.2 precipitate" (22), hydrolyzed tri[^{3}H]-oleoylglycerol at a rate of 4.8 ± 0.2 and 3.3 ± 0.1 nmol/mg × min (n = 3), respectively. Although this is comparable to the rates for HSL reported in the literature (31), our results are overestimates because the pH 5.2 preparation (22) contains 2-monoacylglycerol lipases. The use of the TG analog 1,3-diacyl-2-*O*-alkylglycerol as substrate circumvents this difficulty since HSL hydrolyzes both the 1- and 3-acyl moieties of the substrate but the contaminating monoacylglycerol lipases cannot hydrolyze the remaining 2-(9-octadecenyl)glycerol (32,33). The HSL preparation from epididymal and perirenal tissue hydrolyzed *rac*[^{3}H]oleoyl-[^{14}C]linolenoyl-2-*O*-(9-octadecenyl)glycerol at rates of 1.29 ± 0.01 and 1.8 ± 0.1 nmol/mg × min (n = 3), respectively. Figure 5 shows the proportions of radiolabeled oleate and α-linolenate released from the TG analog substrate. The molar ratio α-linolenate/oleate in the substrate was 1.06 ± 0.01 (n = 4). The enzyme reaction hydrolyzed 3–6% of the total esterified fatty acid during the 30-min incubation period. The epididymal preparation released 35% and the perirenal preparation released 67% more α-linolenate than oleate. The α-linolenate/oleate ratio in the remaining TG analog substrate (Fig. 5) did not change perceptibly because the total amount of fatty acid released was small compared to the total esterified fatty acid present in the reaction mixture.

DISCUSSION

We have used three different and mutually independent models, namely, intact perfused tissue, isolated cultured cells and semi-purified enzyme, representing increasing degrees of organizational complexity. The results from each model show that HSL-mediated TG hydrolysis

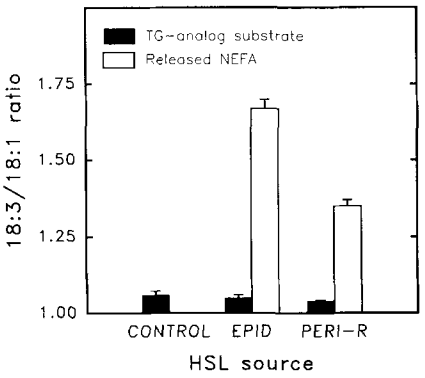

FIG. 5. Preferential hydrolysis of 18:3 by "pH 5.2-precipitate" preparations of hormone-sensitive lipase. The enzyme preparations from peri-renal or epididymal fat pads catalyzed the hydrolysis of the synthetic triglyceride (TG)-analogue substrate *rac*-[^{3}H]oleoyl-[^{14}C]linolenoyl-2-*O*-(9-octadecenyl)glycerol. The ratios of 18:3 and 18:1 in released NEFA were compared with the corresponding ratios in remaining esterified fatty acid. The results represent mean ± SEM of three experiments.

preferentially releases PUFA, regardless of the experimental conditions used. The data indicate that the relase of PUFA is "preferential" and not stringent as it would be in the classic sense of enzyme specificity. Rather, the heterogeneity of TG molecules in adipose gives rise to a situation where the enzyme HSL is confronted with multiple analogous but kinetically different substrates. The results are therefore best explained by postulating that the configuration of TG with PUFA at either the *sn*-1 or *sn*-3 of the glycerol backbone gives it a "kinetic advantage" over the more saturated TG species. Such preference based on degree of unsaturation of the fatty acid moiety has been observed for other lipid enzymes (34,35).

It must be noted that the release of NEFA from adipose is the result of the concerted action of HSL and the re-esterification pathway (36,37). The latter may also be a factor in the preferential release of PUFA, if it re-esterifies saturated fatty acids more than PUFA back into adipose TG. However, others have shown that the extent of release of NEFA from adipose is primarily a function of the availability of fatty acid-poor serum albumin perfusing through the tissue (38–40). Hence, the re-esterification pathway becomes less important when enough carrier is available to trap the released NEFA and keep it away from the adipocyte. We have not fully resolved this point in our experiments, but the use of 2 to 4% fatty acid-free albumin in our experiments should favor the release more than the re-esterification of NEFA in both the pre-adipocyte model and the perfused fat pad model. In contrast, the HSL preparation model is not affected by the re-esterification of NEFA, primarily because of the absence of free CoA in the HSL assay mixture. The results from the latter model reinforce the hypothesis that preferential release of PUFA from the perfused fat pad and from the cultured cells is due at least in part to the preference of HSL for PUFA. It may be argued from the data that adipose HSL does not exhibit dramatic preference for PUFA, and therefore may be of little consequence. While this may be true for the acute short-term experiments described in this report, the *in vivo* situation involves a continuous and multiple-pass perfusion of the fat depots. Thus, preferential release of PUFA observed in an acute experiment represents a high rate of PUFA loss from adipose relative to saturated fatty acids, when considered in the physiological context. It is interesting to note that PUFA-containing chylomicron TG exhibits resistance to hydrolysis by lipoprotein lipase (41). Thus, HSL and lipoprotein lipase act in an opposite but complementary fashion to prevent PUFA from being trapped in fat depots.

The HSL-mediated preferential release of PUFA from adipose TG raises an interesting possibility concerning the control of the positioning of fatty acids on the TG glycerol backbone. It is generally accepted that the 2-acyl moiety of TG tends to be unsaturated while the 1- and 3-acyl moieties tend to be saturated (42,43). This phenomenon has not yet been adequately explained apart from the necessary postulate that the lysophospholipid acyltransferase be specific for PUFA. Thus, the product phosphatidic acid which can either be further processed to TG or to phospholipid, would already contain an unsaturated fatty acid esterified to *sn*-2 of glycerol. However, Christie and Vernon (42) reported that the positional distributions of fatty acids on the glycerol backbone of rat adipose TG is not controlled solely by fatty acid availability nor by acyltransferase specificity. In the extreme case where the esterification of different fatty acids on the glycerol backbone is entirely at random, the preferential hydrolysis of PUFA from the *sn*-1 or *sn*-3 positions by HSL would eventually result in significant accumulation of TG species with PUFA at *sn*-2 of the glycerol backbone. If this alternative mechanism was generally true, then HSL from pig adipose which contains a high proportion of palmitate esterified at the *sn*-2 of the TG-glycerol backbone (42) should preferentially release saturated fatty acids instead of PUFA. We will verify this in future experiments.

In conclusion, we have shown that rat adipose HSL preferentially releases PUFA from TG. This may be an important mechanism in the control of adipose fatty acid composition and the maintenance of a low PUFA content relative to other organs and tissues in the body. This may also be a mechanism by which essential PUFA are kept from being trapped in fat depots and thus maintained in the circulation.

ACKNOWLEDGMENT

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Production of the Criegee Ozonide During the Ozonation of 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine Liposomes

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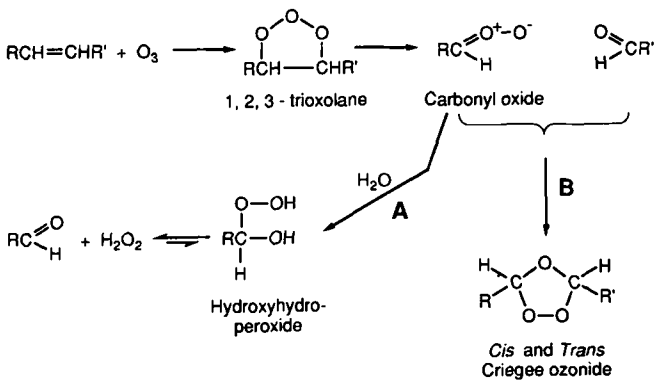
It is likely that Criegee ozonides are formed in small amounts in the lungs of animals breathing ozone-containing air. This makes these compounds potential candidates to act as secondary toxins which relay the toxic effects of ozone deeper into lung tissue than ozone itself could penetrate. Therefore, we have determined the yields of Criegee ozonides from unsaturated lipids in liposomal systems as a model of the types of yields of Criegee ozonides that might be expected both in the lung lining fluid layer and in biological membranes. Ozonation of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine liposomes produced both *cis*- and *trans*-Criegee ozonides. These ozonides have been isolated by solid phase extraction and high-performance liquid chromatography of the ozonized lipid, and the products have been identified by two-dimensional ^1H nuclear magnetic resonance. The combined yield of the *cis*- and *trans*-Criegee ozonides is 10.7 ± 2.8% (avg. ± SD, n = 7) with small unilamellar liposomes and 10.6 ± 2.7% (n = 3) with large multilamellar liposomes. We had previously reported (*Chem. Res. Toxicol. 5*, 505–511, 1992) that ozonation of methyl oleate in sodium dodecylsulfate micelles also produces an 11% yield of the Criegee ozonides. Thus, ozonation in a variety of models gives about 11% of the Criegee ozonide, suggesting that these products also would be formed in small but significant amounts in the lungs of animals breathing polluted air. Further research on the pharmacokinetics and possible toxicity of the Criegee ozonides of fatty acids is suggested.
***Lipids* 27, 955–958 (1992).**

Primary targets for ozone, a highly reactive oxidant present in polluted air, are thought to include the unsaturated fatty acids in the lipids of the lung lining fluid layer and in biological membranes (1–5). Little is known, however, about the mechanisms of ozone toxicity and especially about those steps involved in transmitting the toxicity to distant tissues that ozone itself cannot reach (5).

The ozonation of unsaturated fatty acids gives a 1,2,3-trioxolane, which rapidly decomposes to give a carbonyl oxide and an aldehyde (Scheme 1). In nonparticipating organic solvents, these two species recombine to give the Criegee ozonide (6,7) (Scheme 1, path B). If water is present, the carbonyl oxide can react to give hydroxyhydroperoxides and, ultimately, hydrogen peroxide and a second mole of a carbonyl compound (8–10) (Scheme 1, path A).

The Criegee ozonide, which is relatively stable, must be placed on the list of compounds that could relay the toxic effects of ozone to distal sites. Thus, it is important to determine the yields of Criegee ozonides in systems that model the lung lining fluid layer and cellular membranes, sites where the ozonation of unsaturated fatty acids might occur



SCHEME 1

in vivo. Although Criegee ozonide formation might be inferred from the vast literature available in ozonation in nonparticipating organic solvents (11,12), the yields of Criegee ozonides have not been determined in liposomal systems. We give yields of the ozonides from the ozonation of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in two types of liposomes.

MATERIALS AND METHODS

Chemicals were obtained from the following sources: POPC (Avanti Polar Lipids, Alabasater, AL); choline chloride and sodium phosphate dibasic heptahydrate (Mallinckrodt, Paris, KY); diethylenetriaminepentaacetic acid (DTPA) (Sigma, St. Louis, MO); potassium indigotrisulfonate (Aldrich, Milwaukee, WI); potassium iodide (EM Science, Gibbstown, NJ); and high-performance liquid chromatography (HPLC) grade water and potassium phosphate monobasic (J.T. Baker, Phillipsburg, NJ). Supelclean LC-18 solid phase extraction tubes (3 mL capacity) were purchased from Supelco (Bellefonte, PA).

Generation of ozone. Ozone was generated by passing dried air or oxygen at a constant flow through a Welsbach (Philadelphia, PA) Model T-23 at a fixed voltage (90 V), or an OREC (Phoenix, AZ) O3V-O Series, ozonator producing a silent electric discharge at a fixed amperage (2 amps). Ozone production was estimated iodometrically (13) or by an indigo bleaching method (14).

CAUTION: Due to the possibility of explosion, all ozonation mixtures should be handled with gloves behind a safety shield.

Synthesis of POPC ozonides. A solution of 132 μmoles of POPC in 10 mL of chloroform was dried in a rotary evaporator at 40°C. The dry lipid was dissolved in 100 mL of anhydrous methylene chloride, chilled to −78°C in a dry ice/acetone mixture, and then treated with a stream of ozone (4 μmol/min) in air (60 mL/min) for 30 min. The contents were then brought to room temperature, any unreacted ozone was expelled by a stream of nitrogen, the solvent was evaporated under vacuum, and the residue dissolved in about 3 mL of methanol.

The *cis*- and the *trans*-POPC ozonides in the above sam-

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Abbreviations: DTPA, diethylenetriaminepentaacetic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PB, phosphate buffer; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, SDS, sodium dodecylsulfate.

ple were purified by HPLC according to the method of Lai *et al.* (15) with minor modifications. Aliquots (100 μL each) were chromatographed on a Hypersil ODS (200 × 4.6 mm) column using a mobile phase that consisted of 0.05 M choline chloride in methanol/water (95:5, vol/vol) at a flow of 1.5 mL/min. A Perkin-Elmer (Norwalk, CT) series 410 liquid chromatograph pump equipped with a Perkin-Elmer LC-95 UV/Vis detector was used. The absorbance of the eluent was monitored at 212 nm.

Preparation of liposomes. Small unilamellar liposomes were prepared using the standard sonication method described previously (16,17). Briefly, solutions of 100 mg each of POPC in chloroform (10 mg/mL) were dried in a rotary evaporator at 40°C in a 125-mL pear-shaped flask, giving a thin film of lipid at the bottom half of the flask. A solution of 0.1 M sodium/potassium phosphate buffer (PB, pH 7.4) containing 0.2 mM DTPA was then added to give a final lipid concentration of 2 mM. The lipid film was peeled off and dispersed into the solution by rotating the flask slowly in a L&R sonication bath (Kearny, NJ) Transister/Ultrasonic (T-14 B), yielding large multilamellar liposomes. To prepare small unilamellar liposomes, the milky suspension of large multilamellar liposomes was transferred to a separate beaker and sonicated 3 × 2 min (at intervals of 10 min each) at 0-4°C using a Branson (Danbury, CT) 450 sonifier at full power and 50% duty cycle. The resulting suspension of small unilamellar liposomes was diluted 4-fold with 0.10 M PB, pH 7.4 and allowed to stand at room temperature for 30-60 min before use.

Ozonation of liposomes. Aqueous solutions of ozone were prepared by bubbling a stream of about 1% ozone in oxygen into water that was previously acidified with H_2SO_4 to a pH of 1.95-2.00 (16). The concentration of ozone in solution was estimated after bubbling for 5-10 min by allowing a 1-mL aliquot to react with 1 mL of 0.5 mM indigo trisulfonate in 0.02 M phosphoric acid. The amount of dye bleaching was determined by measuring the decrease in absorbance at 600 nm (ε = 20,000 M^{-1} cm^{-1}) (14). The concentration of ozone in solutions prepared as above ranged between 0.21 and 0.24 mM. Equal volumes (50-100 mL) of ozone solution and liposomal suspension were mixed with stirring and allowed to stand at room temperature for 5 min before further analysis, giving a final pH of about 7.2.

The extent of ozonation of phospholipid in the liposomal suspensions was determined by HPLC with a 20-μL aliquot using a Hewlett-Packard (Avondale, PA) microbore column (200 × 2.1 mm) and a mobile phase consisting of 0.03 M choline chloride in methanol/water (97:3, vol/vol) at a flow of 0.5 mL/min. The eluent was monitored at 205 nm.

Solid phase extraction of ozonized POPC from liposomal ozonation. Supelclean LC-18 3 mL capacity columns were conditioned first with 2 mL of methanol and then with 2 mL of 0.05 M phosphate buffer (prepared by diluting 0.1 M PB, pH 7.4 with H_2SO_4, pH 1.95-2.00). Samples of ozonized liposomes (50-200 mL) were loaded onto the column at a flow of 1.5-2 mL/min. The column was then washed with 3 × 2 mL each of 0.05 M PB and water. The trapped lipids were desorbed with 3 × 2 mL of methanol and the solvent evaporated under a stream of nitrogen.

Purification of POPC ozonides from liposomal ozonations. Phospholipid ozonides in the eluents were purified by HPLC as described above. The fractions with retention times identical to those of synthetic POPC ozonides were collected from several runs. The pooled fractions were evaporated under a stream of nitrogen, the moist residue was resuspended in about 100 mL of water, and subjected to solid phase extraction using a Supelclean LC-18 column. The ozonides were desorbed with 3 × 2 mL of methanol, the solvent evaporated under a stream of nitrogen, and then the ozonides freeze-dried to remove any residual water.

Quantitation of the yield of POPC ozonides in liposomal ozonations. The yield of POPC ozonides in the liposomal reaction was calculated by estimating the phosphorus content of the HPLC fractions corresponding to the unreacted POPC and the POPC ozonides. The fractions were evaporated to dryness under a stream of nitrogen at 60-70°C, and the amount of phosphorus in the residue was estimated according to the method of Ames (18). The relative yields of *cis*- and *trans*-ozonides were calculated by integrating the area under the curves in the HPLC chromatograms of the crude, solid phase extracted POPC ozonation mixtures.

^1H nuclear magnetic resonance (NMR) experiments. Spectra were recorded on a Bruker AM 400 spectrometer (Bruker Spectrospin, Billerica, MA) operating at 400.13 MHz using $CDCl_3$ as solvent. The 2D ^1H-^1H correlation spectroscopy (COSY) spectra were measured at 25°C employing the COSY.AU microprogram of the Bruker software. The spectra were obtained with a digital resolution of 5.425 Hz after zero filling. Zero filling (one) was done in the F1 dimension of a 512 × 512 matrix, the data were 2D transformed, and the magnitude spectra multiplied by a sine window in each dimension and symmetrized along the diagonal.

RESULTS

The solid phase extraction protocol described here allows for a rapid extraction of ozonized lipids from dilute aqueous microemulsions. The ^1H NMR COSY spectra of the *cis*- and *trans*-POPC ozonides obtained by ozonation of POPC in methylene chloride are presented in Figure 1. The resonance assignments are based on the connectivities provided by the proton scalar coupling constants. The *cis*- and *trans*-POPC ozonides show very similar ^1H nuclear magnetic resonance (NMR) spectra except for the resonances at 5.18 and 5.14 ppm that correspond to their respective 1,24-trioxolane ring protons (15,19,20). Other resonances are listed below using the following notation for the H-atoms: H_m-n: where m denotes the moiety within the POPC molecule and can be g (glyceroyl), p (palmitoyl), o (oleoyl), or c (choline), and n denotes the C-atom number to which the H-atom is bonded. The numbering systems used are the standard numbering system for the fatty acid chains and the numbering system of *sn*-glycero-3-phosphocholine for the glycerol moiety. In the case of choline, the numbering starts next to the phosphoryl moiety. POPC ozonides ^1H NMR ($CDCl_3$) δ 5.23 ppm (*m*, 1H, H_g-2); 4.41 ppm (*dd*, 1H, H_g-1); 4.33 ppm (*m*, 2H, H_c-1); 4.12 ppm (*dd*, 1H, H_g-1′); 3.96 ppm (*m*,2H, H_g-3); 3.81 ppm (*m*, 2H, H_c-2); 3.37 ppm (*s*, 9H, H_c-Me); 2.27 ppm (*m*,4H, H_p-2,H_o-2); 1.66 ppm (*m*, 4H, H_o-8,H_o-11); 1.57 ppm (*m*, 4H, H_p-3,H_o-3); 1.40 ppm (*m*, 4H, H_o-7, H_o-12); 1.33-1.17 ppm (*m*, 40H, H_p-4 to H_p-15,H_o-4 to H_o-6,H_o-13 to H_o-17); 0.87 ppm (*t*, 6H, H_p-16,H_o-18). The

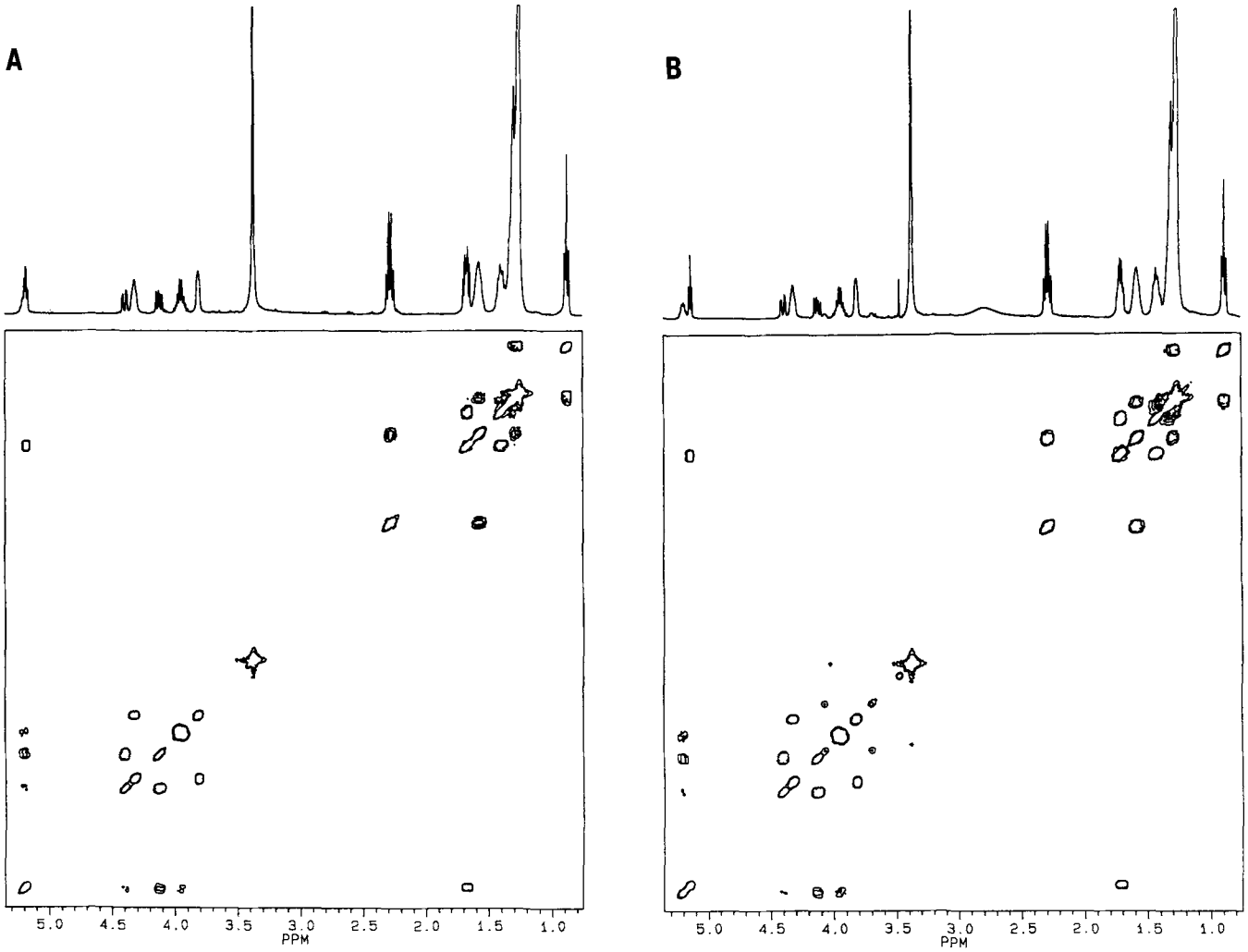

FIG. 1. ^1H-^1H correlation spectroscopy 2D nuclear magnetic resonance spectra of (A) *cis*- and (B) *trans* 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine Criegee ozonides.

POPC ozonides formed by ozonation of POPC liposomes were identified by comparing their ^1H NMR spectra to those of standard samples prepared by ozonating POPC in dry methylene chloride, reaction conditions that we have found to afford high yields of ozonides. A comparison of the ^1H NMR characteristic resonances of the 1,2,4-trioxolane moiety of *cis/trans* mixtures of POPC ozonides obtained by ozonation of POPC liposomes or methylene chloride solutions is shown in Figure 2, confirming that they are indeed the same products (formed in a slightly different *cis/trans* ratio).

DISCUSSION

The average yield of the sum of both the *cis*- and *trans*-POPC ozonides from the ozonation of POPC small unilamellar liposomes is 10.7 ± 2.8% (avg. ± SD, n = 7) and for large multilamellar liposomes is 10.6 ± 2.7% (n = 3). Ozonation conversions were carried to 28–49% in order to obtain sufficient samples for HPLC detection and NMR analysis (Table 1).[1] The POPC ozonides are formed in approximately identical *cis/trans* ratios in the two types of liposomes: 0.86 ± 0.12 (n = 7) and 1.02 ± 0.08 (n = 3) in small unilamellar and large multilamellar liposomes, respectively. This suggests that both the ozonides yield and their *cis/trans* ratios are independent of the type of liposomal preparation.

We have recently reported that Criegee ozonides are formed in about 11% yield when methyl oleate is ozonized in aqueous sodium dodecylsulfate (SDS) micelles (21), a system in which water can penetrate more deeply into the lipid microphase than in the liposomal lipid bilayers reported here. Thus, approximately 10% of the fatty acid is converted to the Criegee ozonide in all three of these model systems: SDS micelles, large multilamellar, liposomes, and small unilamellar liposomes. Since these systems differ in their degree of hydration (22,23) but afford very similar yields of Criegee ozonides, it seems reasonable to extrapolate a similar yield of Criegee ozonides in the lining fluid layer and/or cellular membranes in the lungs of animals exposed to ozone.

Cortesi and Privett (24) found that methyl linoleate ozonides injected into the tail vein were lethal to rats at 0.07 nnol/100 g body weight. Although these workers report that damage occurs primarily in the lung of the in-

[1] At these conversions, it is possible that the liposomal structure is altered by the presence of broken and oxygenated fatty acid chains.

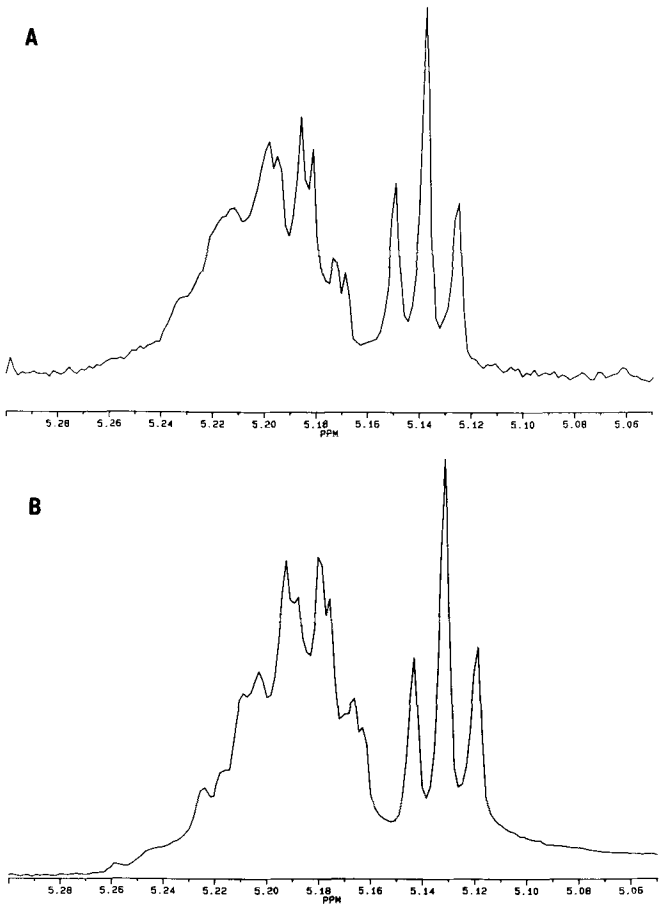

FIG. 2. Comparison of the ^1H nuclear magnetic resonance characteristic resonances of the 1,2,4-trioxolane moiety of *cis/trans* mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) ozonides obtained by ozonation of (A) POPC liposomes or (B) methylene chloride solutions.

TABLE 1

Formation of Criegee Ozonides from the Ozonation of POPC Liposomes[a]

% POPC ozonized	% Yield of ozonides	*cis/trans* Ratio
39[b]	9.4	0.98
41[b]	8.9	0.63
36[b]	16.0	0.91
37[b]	12.8	1.00
43[b]	8.4	0.81
38[b]	9.1	0.84
43[b]	10.0	0.87
28[c]	13.7	1.11
37[c]	9.2	0.98
49[c]	8.8	0.97

[a]Values presented are means of analyses carried out in either duplicates or triplicates. The variation among replicates was less than 5% in all cases. POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.
[b]Small unilamellar liposomes.
[c]Large multilamellar liposomes.

jected rats, this does not appear to be the optimum approach to model the situation that would exist in the lung when ozone-containing air is breathed. In that situation, the Criegee ozonide would be formed *in situ* in the lung lining fluids and/or bilayers. It is known that the Criegee ozonide can initiate lipid peroxidation, although at slow rates (19). Thus, the Criegee ozonide is of some interest as a potential secondary toxin from ozone exposures, underlining the need for more detailed investigations of the formation and toxicity of ozonides *in vivo*.

The hydrolytic stability of Criegee ozonides in carbon tetrachloride and in SDS micelles at physiological pH is quite good, with lifetimes in the order of several days (19, 21); however little is known about the half-life, metabolism and clearance times for Criegee ozonides in biological systems. Clearly, some data on the pharmacokinetics of the Criegee ozonide is needed, and we have initiated a program to obtain the relevant data. Additionally, because Criegee ozonides are expected to be formed only in reactions with ozone, these compounds might serve as useful biological marker molecules for ozone toxicity.

ACKNOWLEDGMENTS

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Kinetics of Photoperoxidation of Arachidonic Acid: Molecular Mechanisms and Effects of Antioxidants

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The kinetics of photoperoxidation of [1-^{14}C]arachidonic acid (20:4n-6) at 1.32 mM was studied either with the unsaturated fatty acid alone or in the presence of 10 μM of antioxidants and/or inhibitors of eicosanoid metabolism. The photosensitizer used was *meso*-tetraphenylporphine. The time-course of the reactions was followed by ultraviolet spectral analysis, thiobarbituric acid reactivity and high-performance liquid chromatographic analysis of aliquots sampled every 15 min during the 4 h of irradiation. The kinetics of photoperoxidation of 20:4n-6 can be divided into three main successive steps: (i) monohydroperoxidation, characterized by the appearance of conjugated diene patterns and monohydroperoxidized 20:4n-6; (ii) secondary oxidation characterized by polyoxygenated products such as dihydroperoxidized 20:4n-6 possessing conjugated triene patterns; and (iii) the disappearance of conjugated patterns and the oxidative cleavage of the products of the two first steps into aldehydic molecules reacting with thiobarbituric acid. During the first 90 min of irradiation, the mechanism of monohydroperoxidation (step one) is purely or predominantly type II photoperoxidation involving only singlet oxygen. This step was inhibited by β-carotene and by BW755C (3-amino-1-[3-trifluoromethylphenyl]2-pyrazoline). In contrast, the reactions involved in the second and third steps were predominantly type I photoperoxidation involving radical mechanisms. These latter steps were inhibited by β-carotene, BW755C, vitamin E and probucol. Indomethacin and 5,8,11,14-eicosatetraynoic acid did not alter 20:4n-6 photoperoxidation. This *in vitro* model of lipid photoperoxidation allows the screening of antioxidants in accordance with their singlet oxygen quenching and/or free radical scavenging properties.
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Nonenzymatic peroxidation of unsaturated membrane lipids has been induced using photochemical processes in numerous *in vitro* and *in vivo* studies of basic and/or pharmacological relevance (1-6). Lipid photoperoxidation is of obvious significance as a model for the study of diseases involving photoreactive processes, such as porphyrias or cataracts (7–9). However, the relevance of photochemical processes to the study of "dark" oxidative stress, such as that generated during cardiac or cerebral ischemia-reperfusion injury remains less certain. Indeed, the photoperoxidation of unsaturated lipids involves both nonradical peroxidation by singlet oxygen (type II photoperoxidation) and radical peroxidation by free radicals derived from a photosensitizer (type I photoperoxidation) (10,11). The nature of the photosensitizer is one of the factors which determines the relative balance between these two mechanisms (12). The participation of singlet oxygen in "dark", nonenzymatic peroxidation of lipids has been suggested by numerous authors (13–15). However, there is no clear proof that "dark" generation of singlet oxygen occurs *in vivo*.

We wished to compare the pharmacological sensitivity of nonenzymatic lipid peroxidation occurring *via* free radical processes and nonradical processes involving singlet oxygen. In this report, we describe experiments in which the kinetics of photoperoxidation of the polyunsaturated lipid arachidonic acid (20:4n-6) was studied with the unsaturated fatty acid alone, or in the presence of one of the six drugs described hereafter. Each drug tested displays a unique activity profile as an antioxidant and/or as an inhibitor of eicosanoid metabolism. Vitamin E (VitE) is the main physiological antioxidant (9). Probucol (PROB; 2,2-*bis*[3,5-di-*t*-butyl-4-hydroxyphenylthio]propane) is a free radical scavenger used for the inhibition of the oxidation of low density lipoproteins probably involved in atherosclerosis (16,17). β-Carotene (βCAR) is physiologically relevant both as a free radical scavenger and as a singlet oxygen quencher (18,19). BW755C (3-amino-1-[3-trifluoromethylphenyl]2-pyrazoline) is a dual inhibitor of cyclooxygenase and lipoxygenase acting *via* a free radical mechanism of inhibition of these enzymes (20). BW755C protects the heart against oxidative stress associated with ischemia-reperfusion injury (21–23). ETYA (5,8,11,14-eicosatetraynoic acid) is an analogue of 20:4n-6 and a competitive inhibitor of both cyclooxygenase and lipoxygenase (24). Indomethacin (INDO) is an inhibitor of cyclooxygenase (24).

MATERIALS AND METHODS

Materials. Unlabelled 20:4n-6 and eicosapentaenoic acid (20:5n-3), βCAR, INDO, *meso*-tetraphenylporphine (mTPP), soybean lipoxygenase and VitE were obtained from Sigma Chemical Co. (St. Louis, MO). ETYA was from Fluka (Buchs, Switzerland). The nonlabelled eicosanoids employed as standards were purchased from Cayman Chemicals (Denver, CO). [1-^{14}C]20:4n-6 (specific activity 2.2 GBq/mmol) and [1-^{14}C]20:5n-3 (specific activity 1.9 GBq/mmol) were obtained from Amersham (Cardiff, U.K.) as toluene solutions containing no antioxidant. BW755C and PROB were from Wellcome (London, U.K.) and Merrell Dow (Cincinnati, OH), respectively. All organic solvents and alcohols used were high-performance liquid chromatography (HPLC) grade (Farmitalia Carlo Erba,

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Abbreviations: AU, absorbance unit; BHT, butylated hydroxytoluene; βCAR, β-carotene; CP-HPLC, chiral-phase high-performance liquid chromatography; DiHETEs, dihydroxyeicosatetraenoic acids; DiHpETEs, dihydroperoxyeicosatetraenoic acids; ETYA, 5,8,11,14-eicosatetraynoic acid; 15(*S*)-HEPE, 15(*S*)-hydroxyeicosapentaenoic acid; HETEs, hydroxyeicosatetraenoic acids; 15(*S*)-HpEPE, 15(*S*)-hydroperoxyeicosapentaenoic acid; HpETEs, hydroperoxyeicosatetraenoic acids; INDO, indomethacin; MDA, malondialdehyde; mTPP, *meso*-tetraphenylporphine; NP-HPLC, normal-phase high-performance liquid chromatography; PROB, probucol; RP-HPLC, reverse-phase high-performance liquid chromatography; TBARS, thiobarbituric acid-reactive substances; TFA, trifluoroacetic acid; UV, ultraviolet; VitE, vitamin E.

Milano, Italy). All others chemicals were analytical grade and were obtained from Merck (Darmstadt, Germany). All chemicals were used as received, with the exception of 20:4n-6 and 20:5n-3 which were purified as described below.

Purification of 20:5n-3 and labelled 20:4n-6. Isotopic dilution of [1-^{14}C]20:4n-6 (12.4 KBq/μmol) was accomplished by mixing 2.05 MBq of labelled 20:4n-6 (2.2 GBq/mmol) with 1645 μmol (50 mg) of nonlabelled 20:4n-6. Because of the great sensitivity of 20:4n-6 to spontaneous autoxidation in the presence of air, the dilution of [1-^{14}C]-20:4n-6 (12.4 KBq·μmol^{-1}) was purified less than 5 d prior to experiments to eliminate autoxidation products. Briefly, 50 mg of labelled 20:4n-6 were passed through a column packed with 1 g of silicic acid Silicar CC-4 (Mallinckrodt, Paris, KY) using hexane/diethyl ether (92:8, vol/vol) as mobile phase. Only the first 35 mL of eluate were collected. After rotary evaporation at 20°C, a methanolic solution of purified 20:4n-6 was prepared. The purity of 20:4n-6 was confirmed by ultraviolet (UV) spectroscopy and reverse-phase high-performance liquid chromatography (RP-HPLC) in combination with radioactive detection as described below. The concentration of the methanolic solution of 20:4n-6 was measured spectrophotometrically using an experimentally determined molar extinction coefficient of 17130 absorbance unit (AU)·cm^{-1}·M^{-1} at 204 nm. Eicosapentaenoic acid was purified as for 20:4n-6 using a molar extinction coefficient of 21400 AU·cm^{-1}·M^{-1} at 204 nm. The purified solutions of 20:5n-3 and labelled 20:4n-6 were stored at −85°C.

Preparation of [1-^{14}C]15(S)-hydroxyeicosapentaenoic acid. The isotopic dilution of [1-^{14}C]20:5n-3 (11.2 KBq/μmol) was prepared by adding 370 KBq of [1-^{14}C]-20:5n-3 (1.9 GBq/mmol) to 10 mg of purified 20:5n-3. [1-^{14}C]15(*S*)-hydroperoxyeicosapentaenoic acid (15(*S*)-HpEPE) was synthetized by incubating 10 mg of isotopically diluted [1-^{14}C]20:5n-3 as sodium salt with 7 mg of soybean lipoxygenase for 10 min at 25°C in 250 mL of *tris*(hydroxymethyl)aminoethane/HCl buffer (50 mM, pH 9). After acidification, 15(*S*)-HpEPE was extracted with diethyl ether. [1-^{14}C]15(*S*)-hydroxyeicosapentaenoic acid ([1-^{14}C]15(*S*)-HEPE) was obtained by reduction of the dried diethyl ether extract resolubilized in 50 mL of methanol with 15 mg of $NaBH_4$ for 120 min at 0°C. After drying and addition of acidified water, [1-^{14}C]15(*S*)-HEPE was extracted with diethyl ether. [1-^{14}C]15(*S*)-HEPE was purified from the diethyl ether acidic extract by isocratic normal-phase HPLC (NP-HPLC) using a 350 mm × 7.5 mm Nucleosil 120-10 column (Macherey-Nagel, Düren, Germany). The column was eluted at a constant flow of 6 mL/min (Waters 590 pump, Millipore, Milford, MA) with a mobile phase consisting of *n*-heptane/ethanol/acetic acid (995:4:1,vol/vol/vol). The effluent was monitored with a UV detector (Waters 481, Millipore) at 235 nm. The retention time of [1-^{14}C]15(*S*)-HEPE was 26 min. [1-^{14}C]15(*S*)-HEPE was collected at the outlet of the UV detector. [1-^{14}C]15(*S*)-HEPE was identified by comparison of its retention time with that of nonradioactive commercial 15(*S*)-HEPE and was quantified by UV analysis using 23000 AU·cm^{-1}·M^{-1} as molar extinction coefficient at 235 nm. [1-^{14}C]15(*S*)-HEPE was stored at −85°C in methanol.

Photoperoxidation procedure. The reactions were performed at 25°C in the apparatus illustrated in Figure 1.

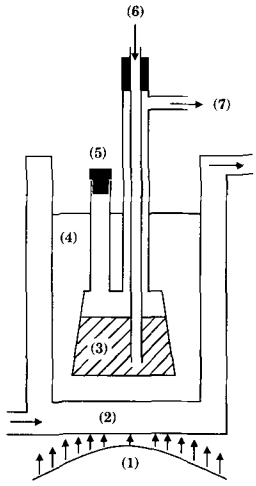

FIG. 1. Apparatus for the photoperoxidation experiments. The reaction vessel and the cooling jacket were constructed from glass. (1): 120 W tungsten lamp operated at 24 V; (2): cooling jacket with circulating water at 25°C; (3): reaction vessel containing irradiated and oxygen-bubbled sample; (4): yellow filter-solution K_2CrO_4 (0.1M); (5): sampling tube; (6) and (7): inlet and outlet for O_2 stream at 10 mL/min.

In a typical experiment, 10 mg of labelled and purified 20:4n-6 and 300 μg of mTPP in a final volume of 25 mL in CCl_4 were irradiated in the reaction vessel. The appropriate amount of test drugs was added in 250 μL of ethanol at 0 min or at 135 min of irradiation. The final concentrations were 1.32 mM of 20:4n-6, 17 μM of mTPP and 10 μM of drugs to be tested. The molar ratio of tested drug/oxidizable substrate (20:4n-6) was 0.0076. For the analytical procedures, an aliquot of 500 μL of reaction solution was removed and placed on ice every 15 min during 240 min of irradiation.

UV analysis. A portion of the peroxidized aliquot was treated to obtain 1 mL of methanolic solution at 55 μM equivalent initial concentration of 20:4n-6. UV spectra were obtained using a scanning spectrophotometer (Uvikon 860, Kontron, Zürich, Switzerland). The methanolic solutions were scanned from 190 nm to 320 nm with methanol as background. The conjugated diene and conjugated triene patterns present absorbance maxima between 234-237 nm and at 268 nm, respectively. These specific absorbances were studied because the majority of monohydroperoxides and dihydroperoxides derived from polyunsaturated fatty acids possesses conjugated diene and conjugated triene patterns, respectively (25). The specific absorbances were characterized by analysis of the second derivative of spectra (26,27). The UV spectral results were expressed as mAU/μM equivalent initial concentration of 20:4n-6 at 235 nm and 268 nm as a function of irradiation time.

RP-HPLC analysis. RP-HPLC of peroxidized products was done on a 150 mm × 7.4 mm Nucleosil 120-3 C18 column (Macherey-Nagel). The column was eluted at a constant flow of 1 mL/min for a run duration of 55 min, with two successive isocratic steps: the first 40 min with ethanol/water/sulfuric acid (630:370:0.5, vol/vol/vol) and the last 15 min with ethanol/sulfuric acid (1000:0.5, vol/vol). The effluents were successively monitored in line with a

photodiode array detector (Waters 990, Millipore) between 190 nm and 300 nm and with an HPLC radioactivity detector (Berthold, model LB507A, Wildbad, Germany) using solid scintillation with an yttrium glass cell. The injected samples were prepared from a photoperoxidized aliquot of 400 nmoles of equivalent initial 20:4n-6. This aliquot had been previously stabilized by reduction with 1-2 mg of $NaBH_4$ in methanolic solution for 120 min at 0°C. After rotary evaporation at 20°C, the dry sample was dissolved in 500 μL of an ethanol/water/acetic acid (50:50:20, vol/vol/vol) mixture and filtered through a nylon membrane. The sample volume injected was 250 μL. The results were expressed for each class of peroxidative product as the area under the corresponding peaks as a percentage of the total area of all peaks of radioactivity as a function of irradiation time.

Separation of optical isomers of isolated positional isomers of hydroxyeicosatetraenoic acids (HETEs). The stereochemical analysis of each HETE required prior purification of the positional isomers of HETEs by NP-HPLC. NP-HPLC of HETEs was performed with a 250 mm × 4.6 mm Nucleosil 120-3 column (Macherey-Nagel). The column was eluted at a constant flow of 1 mL/min for a 75 min run. The first 10 min and the last 30 min were isocratic steps with *n*-heptane/2-propanol/trifluoroacetic acid (TFA) (995:5:0.1, vol/vol/vol) and *n*-heptane/2-propanol/TFA (985:15:0.1, vol/vol/vol) as mobile phases, respectively. From 10 min to 45 min, the mobile phase consisted of a linear gradient between the two solvent mixtures described above. The detection mode was identical to that used for RP-HPLC. Ten mg of [^{14}C]-20:4n-6 photoperoxidized during 60 min were reduced with 15 mg of $NaBH_4$ in 50 mL of methanol for 120 min at 0°C before rotary evaporation. The dry samples were washed with 1 M HCl. The reduced products of photoperoxidation were extracted from the acidic solution with diethyl ether. After rotary evaporation, the dry samples were dissolved in 250 μL of injection mixture consisting of *n*-heptane/2-propanol (995:5, vol/vol). Chiral-phase HPLC (CP-HPLC) was performed on a 250 mm × 4.6 mm Chiralpak AD 10 μm column (Daicel, Düsseldorf, Germany) as stationnary phase and with *n*-heptane/2-propanol/TFA (980:20:0.02, vol/vol/vol) as mobile phase at a constant flow of 1 mL/min. The samples were injected in 100 μL of mobile phase; the detection mode was identical to that used for the NP-HPLC procedure.

Thiobarbituric acid-reactive substances (TBARS). The determination of TBARS formation during control photoperoxidation in absence of test drugs was based on procedure as described (28). TBARS were evaluated directly from 500 μL of photoperoxidized samples after acidic aqueous extraction from the CCl_4 phase with 1.5 mL of 0.5 M HCl. The extraction was performed without evaporation steps because it became evident that during rotary evaporation volatile aldehydes can be lost. Butylated hydroxytoluene (BHT) was added to the reaction solution before incubation at 95°C. The addition of BHT inhibits the thermocleavage of lipid hydroperoxydes to aldehydic products. Photoperoxidized aliquots of 400 nmoles of equivalent initial 20:4n-6 were used for TBARS determination. Malondialdehyde (MDA) was employed as calibration standard. TBARS formation was expressed in nmoles MDA/μmole equivalent initial amount of 20:4n-6 as a function of irradiation time.

RESULTS

Characterization of conjugated dienes and trienes. Photoperoxidation of 20:4n-6 during the first 150 min led to rapid, time-dependent formation of conjugated patterns (Fig. 2). The maxima of absorbance spectra of photoperoxidized 20:4n-6 corresponded to the maxima of absorbance spectra of authentic enzymatically-generated standards of conjugated dienes 5(*S*)-hydroxyeicosatetraenoic acid (5(*S*)-HETE) and conjugated trienes 5(*S*),12(*S*)-dihydroxyeicosatetraenoic acid (5(*S*),12(*S*)-DiHETE). These results were confirmed by analysis of the second derivative of the spectra (26,27).

Chromatographic characterization of peroxidized products of 20:4n-6. Figure 3 shows a representative RP-HPLC analysis of labelled 20:4n-6 which was photoperoxidized for 135 min. The distribution of the total radioactivity was 4.1% for residual 20:4n-6 with an elution time of 50 min, 34.4% for intermediate monohydroperoxidized products (hydroperoxyeicosatetraenoic acids, HpETEs) that eluted between 29 and 43 min and 61.5% for polar terminal products. This last group included all the peaks that eluted between 5 and 28 min. In this group, numerous peaks (not formally identified) exhibited conjugated diene and triene patterns. This group included the products of secondary peroxidation such as endoperoxides, cyclic peroxides and dihydroperoxyeicosatetraenoic acids (DiHpETEs) and the aldehydic products of final cleavages of mono and dihydroperoxidized 20:4n-6. $NaBH_4$ reduction of endoperoxides generated dihydroxylated compounds. The

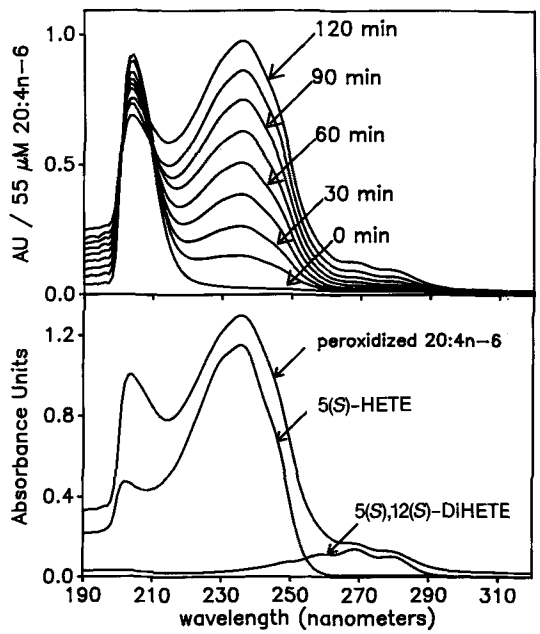

FIG. 2. Appearance and characterization of conjugated patterns during photoperoxidation of 20:4n-6. Upper panel: ultraviolet (UV) spectra of the successive production of conjugated dienes and trienes during the first 120 min of irradiation, one measurement every 15 min. Lower panel: comparison of the UV spectrum of 20:4n-6 photoperoxidized for 120 min with the UV spectra of 5(*S*)-hydroxyeicosatetraenoic acid (HETE) and 5(*S*),12(*S*)-dihydroxyeicosatetraenoic acid (DiHETE). The two molecules possess conjugated diene and conjugated triene patterns, respectively.

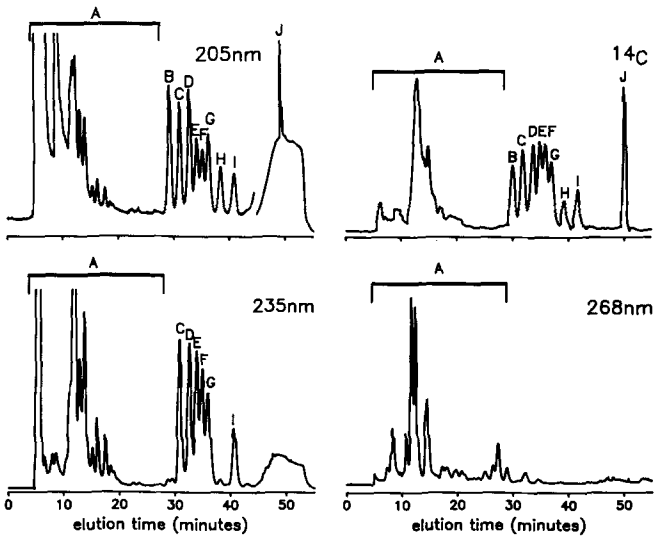

FIG. 3. Reverse-phase high-performance liquid chromatography of [1-^{14}C]20:4n-6 photoperoxidized for 135 min. For the same run, ultraviolet (UV) (205 nm, 235 nm and 268 nm) and radioactive detections were used. The sample was stabilized by reduction with $NaBH_4$. Peaks A: group of terminal products of peroxidation in which were pooled reduced products of secondary oxidation such as dihydroperoxyeicosatetraenoic acid and aldehydic products of the oxidative cleavage of hydroperoxidized 20:4n-6. Peaks B–I represent the reduced hydroperoxyeicosatetraenoic acids as hydroxyeicosatetraenoic acids (HETEs). Peak B: 14-HETE. Peak C: 15-HETE. Peak D: 11-HETE. Peak E: 12-HETE. Peak F: 8-HETE. Peak G: 9-HETE. Peak H: 6-HETE. Peak I: 5-HETE. 14-HETE (peak B) and 6-HETE (peak H) do not possess conjugated diene patterns and do not absorb at 235 nm. Peak J: residual 20:4n-6.

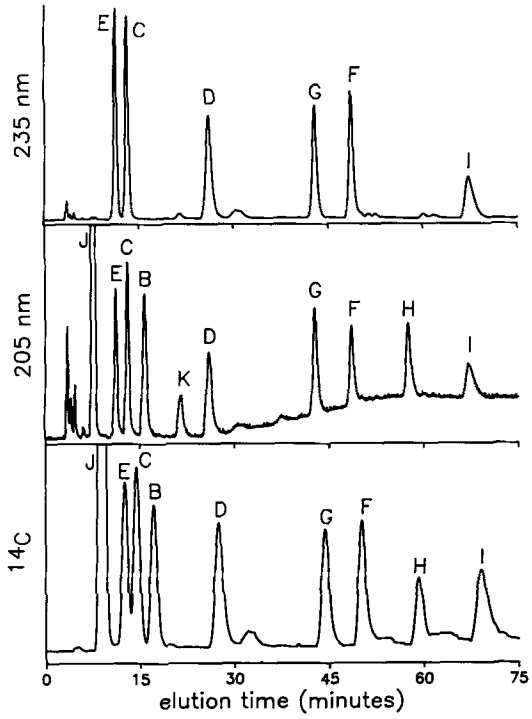

FIG. 4. Normal-phase high-performance liquid chromatography of [1-^{14}C]20:4n-6 photoperoxidized for 60 min. Ultraviolet (205 nm and 235 nm) and radioactive ^{14}C detections were successively used for each run. The sample was stabilized by reduction with $NaBH_4$. The identification of the peaks (B–I) is identical to that used in Figure 3. Peak K: oxidized *meso*-tetraphenylporphine (mTPP).

aldehydic nature of the products generated by cleavage of peroxidized 20:4n-6 was confirmed by the appearance of TBARS. TBARS generation is not a valid index for a dynamic evaluation of the cascade of reactions involved in *in vivo* lipid peroxidation, but it is a relevant index for the global quantification of carbonyl molecules in an *in vitro* model containing no cellular contaminants (carbohydrates and nucleic acids) as described here. The production of HpETEs preceded the appearance of the peaks of the terminal products.

In autoxidation experiments (radical mechanism), only six conjugated positional isomers of HpETEs have been obtained: 15-HpETE, 12-HpETE, 11-HpETE, 9-HpETE, 8-HpETE, 5-HpETE (29,30). In contrast, eight positional isomers of HpETEs have been formed in photoxidation experiments: the six conjugated HpETEs as those obtained in the case of autoxidation and two supplementary nonconjugated products, 14-HpETE and 6-HpETE (Fig. 3). The formation of these two nonconjugated isomers is characteristic of the initiation of peroxidation of 20:4n-6 by singlet oxygen *via* a nonradical mechanism, *i.e.* ene addition (31–33). The positional isomers of HpETEs (measured as HETEs after stabilizing reduction) were identified by comparison of their elution times and of their UV spectra (performed on line with photodiode array detector) with those of standards. The six conjugated HETEs standards were from commercial sources, while 14-HETE and 6-HETE were previously identified using an NP-HPLC procedure illustrated in Figure 4 in agreement with Chacon *et al.* (33). The relative distribution of radioactivity between the eight positional isomers of HpETEs after 90 min of irradiation were 15.5% for 15-HETE, 13.4% for 14-HETE, 12.9% for 12-HETE, 14.2% for 11-HETE, 11.5% for 9-HETE, 14.3% for 8-HETE, 7.9% for 6-HETE and 10.3% for 5-HETE, respectively. These results were similar to those of Chacon *et al.* (33) using methylene blue or haematoporphyrin as photosensitizer. Figure 5 shows four examples of a separation of optical isomers of isolated HETEs produced by photoperoxidation in comparison with the corresponding *S*-isomers. Production of HETEs by photoperoxidation yielded racemic mixtures of *R*- and *S*-isomers.

Time-course of nonenzymatic peroxidation of 20:4n-6. The successive appearance and disappearance of conjugated patterns were indicative of the formation of mono- and dihydroperoxidized compounds followed by their oxidative cleavages to final aldehydic products that do not contain conjugated patterns (Fig. 6). The TBARS generation was strictly linked with the phase of disappearance of conjugated patterns. The absorbance maxima of conjugated dienes and trienes were observed after 150 min and 165 min of irradiation, respectively. The transition from the formation of conjugated patterns to the disappearance of these structures occurred after 165 min of irradiation. At this time, the irradiated solution became opaque, indicating the formation of polar molecules as aldehydic products that are insoluble in CCl_4. The lag period of 60 min before the appearance of conjugated trienes contrasted with the direct formation of conjugated dienes in the early minutes of irradiation and underlined the successive transformation of monohydroperoxidized products to dihydroperoxidized products as described (31).

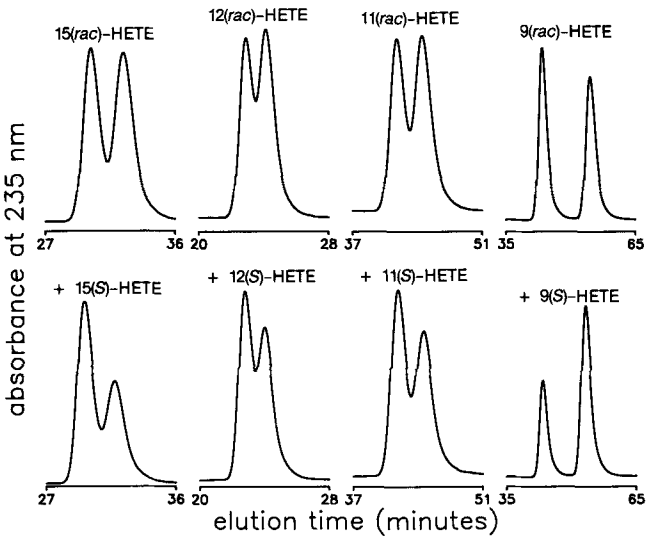

FIG. 5. Chiral-phase high-performance liquid chromatography (CP-HPLC) of separated positional hydroxyeicosatetraenoic acid (HETE) isomers produced by photoperoxidation. Top row: CP-HPLC of four HETEs produced by photoperoxidation. Bottom row: CP-HPLC of these four HETEs produced by photoperoxidation mixed with the corresponding optical *S*-isomers.

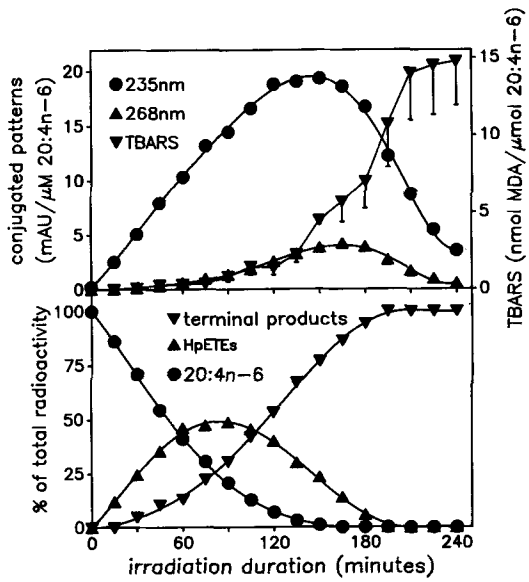

FIG. 6. Time-course of the production of conjugated patterns, thiobarbituric acid-reactive substances (TBARS) and the evolution of classes of peroxidation products during photoperoxidation of [1^{-14}C]20:4n-6 at 1.32 mM. Upper panel: conjugated diene and conjugated triene patterns are expressed as milli absorbance unit (mAU) at 235 nm/μM 20:4n-6 and mAU at 268 nm/μM 20:4n-6, respectively, as a function of irradiation time. The data were obtained from ultraviolet (UV) spectra as described in Figure 2. TBARS are expressed as nmoles of malondialdehyde (MDA) produced by μmole of initial 20:4n-6 as a function of irradiation time. Lower panel: from reverse-phase high-performance liquid chromatography analysis as described in Figure 3, the distribution of total ^{14}C between terminal products of peroxidation (products of secondary oxidation and aldehydic products of the oxidative cleavage of hydroperoxides), hydroperoxyeicosatetraenoic acids (HpETEs) and 20:4n-6 are expressed as a function of irradiation time. The results for the conjugated patterns and the distribution of total ^{14}C were expressed as the mean of eleven experiments; the standard deviations were less than 3% of the mean. For TBARS, the results are expressed as the mean ± SD for four experiments.

The chromatographic results shown in Figure 6 illustrated that: (i) 20:4n-6 is completely peroxidized after 150 min of irradiation; (ii) HpETEs are the early transitional peroxidized compounds formed with a maximum accumulation of 48.7% of total radioactivity after 90 min of irradiation; and (iii) HpETEs are totally transformed to terminal products after 195 min of irradiation; these terminal products, including secondary oxidation products as dihydroperoxides and final aldehydic compounds, accounted for 100% of total radioactivity.

In preliminary experiments, [1^{-14}C]15(*S*)-HEPE was used as an internal standard for RP-HPLC analysis. There was no change in total radioactivity of each aliquot sampled during the 240 min of irradiation (data not shown). This indicated that there was no formation of volatile terminal compounds possessing labelled carbon from the carboxylic function of 20:4n-6 during the 240 min of irradiation.

The initial lag period in the formation of TBARS is identical to that obtained for the formation of conjugated trienes (Fig. 6). TBARS, like the terminal products identified chromatographically, exhibited a plateau beginning after 195 min of irradiation. However, a difference of 60 min was observed between the irradiation times necessary for these two indices of final peroxidation to reach 50% of their maximal values. The formation of chromatographically-identified terminal products preceded TBARS formation.

Effect of Vitamin E. The addition of VitE (10 μM) at the beginning of the irradiation period modified neither the rate of formation of HpETEs and conjugated dienes, nor the time of irradiation necessary to obtain maximal values (Fig. 7). In contrast, VitE added at 0 min decreased the rate of formation of conjugated triene patterns by 43.4% and the rate of disappearance of HpETEs by 55.4%. VitE added at 0 min inhibited totally the phase of decrease of conjugated patterns. Under these conditions, VitE inhibited the later steps of cleavage of mono- and dihydroperoxidized products to terminal aldehydic compounds. To test directly the effects of the antioxidants on the oxidative cleavage of monohydroperoxidized products, drugs were added after 135 min of irradiation. When VitE was added after 135 min of irradiation, the rate of disappearance of HpETEs was diminished by 61.1% and as described above, VitE totally abolished the phase of decrease of conjugated patterns (data not shown).

Effect of Probucol. The addition of PROB (10 μM; data not shown) at the beginning of the irradiation period modified the indices of peroxidation in the same way as VitE. PROB added at 0 min decreased the rate of formation of conjugated triene patterns and the rate of disappearance of HpETEs by 36.7% and 46.1%, respectively.

Effect of BW755C. The addition of BW755C (10 μM) at the beginning of the 240 min irradiation period decreased the formation of HpETEs and conjugated dienes and trienes by 53.5%, 53.4% and 61.2%, respectively (Fig. 8), and the step of disappearance of mono- and dihydroperoxidized products was not observed. The addition of BW755C (10 μM) after 135 min of irradiation decreased the rate of disappearance of HpETEs by 81%, and conjugated dienes

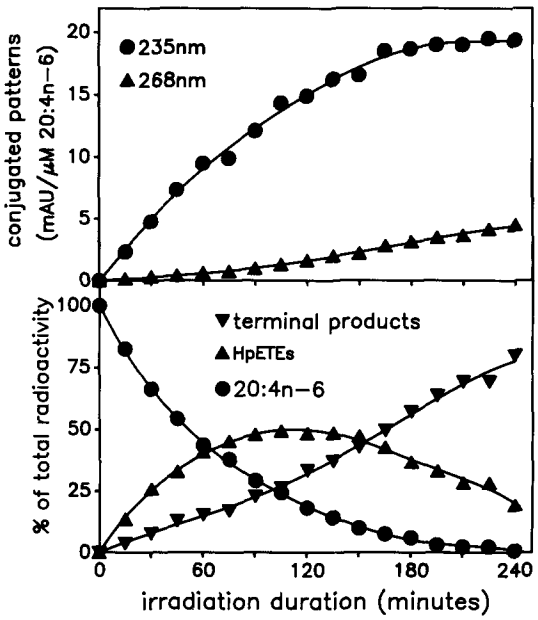

FIG. 7. Effect of Vitamin E (VitE) (10 μM; added at 0 min of irradiation) on the time-course of production of conjugated patterns and evolution of classes of peroxidation products during photoperoxidation of [1^{-14}C]20:4n-6 at 1.32 mM. The molar ratio of tested drug (VitE)/oxidizable substrate (20:4n-6) was 0.0076. The results are expressed as a function of the duration of irradiation for ultraviolet spectral analysis (upper panel) and reverse-phase high-performance liquid chromatography analysis (lower panel) as described in Figure 6. The results represent the mean of three experiments.

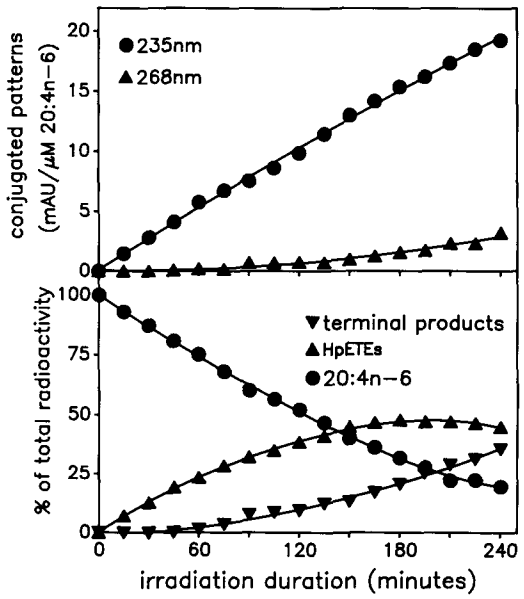

FIG. 8. Effects of BW755C (10 μM; added at 0 min of irradiation) on the time-course of the production of conjugated patterns and the evolution of classes of peroxidation products during photoperoxidation of [1^{-14}C] 20:4n-6 at 1.32 mM. The results are expressed as described in Figure 7.

and trienes were stabilized at maximal levels (data not shown). BW755C was thus able to inhibit both the generation of mono- and dihydroperoxidized products and the subsequent oxidative cleavage of these products.

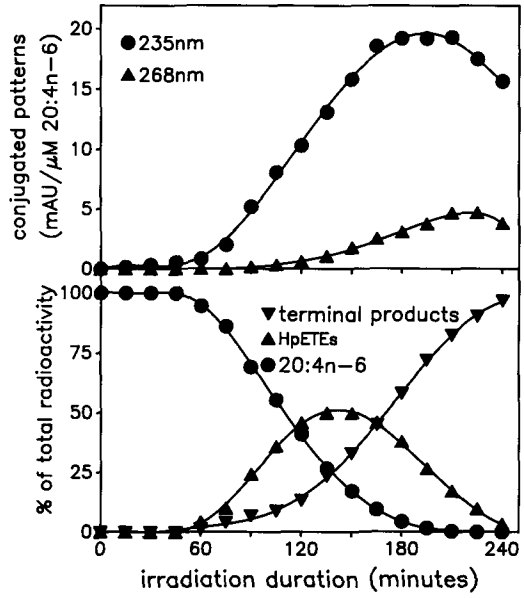

FIG. 9. Effects of beta-carotene (10 μM; added at 0 min of irradiation) on the time-course of the production of conjugated patterns and the evolution of classes of peroxidation products during photoperoxidation of [1^{-14}C] 20:4n-6 at 1.32 mM. The results are expressed as described in Figure 7.

Effect of β-carotene. The addition of βCAR (10 μM) at the beginning of the irradiation period produced a lag period of 60 min prior to the formation of HpETEs and conjugated dienes and trienes (Fig. 9). At the end of this lag period, characterized by the disappearance of the orange color of βCAR, the peroxidation reactions began at rates equal to those observed under control conditions.

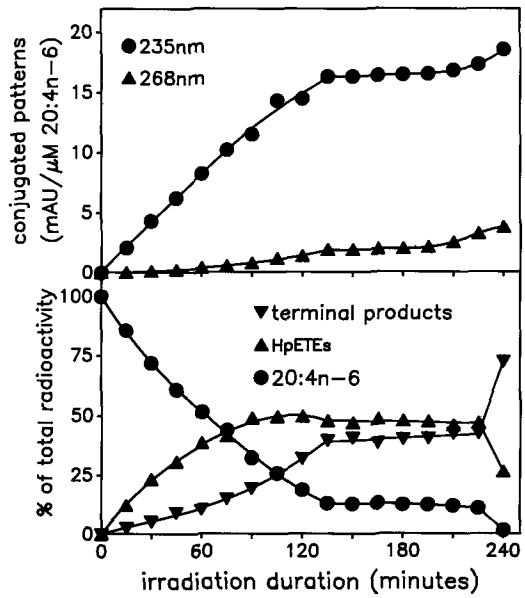

FIG. 10. Effects of beta-carotene (10 μM; added at 135 min of irradiation) on the time-course of the production of conjugated patterns and the evolution of classes of peroxidation products during photoperoxidation of [1^{-14}C]20:4n-6 at 1.32 mM. The results are expressed as described in Figure 7.

The addition of βCAR (10 μM) after 135 min of irradiation stabilized the different parameters at the values observed at 135 min for a period of 90 min (Fig. 10). After 225 min of irradiation, the different reactions began at rates equal to those observed under control conditions. As described above, the resumption of the reactions was associated with the disappearance of the orange carotenoid dye. If in the absence of βCAR, the irradiation was interrupted at 135 min of irradiation and resumed 90 min later, the kinetics of the different indices of peroxidation were similar to those observed with βCAR addition at 135 min (data not shown). These results indicated that βCAR protected the lipids from photoperoxidation by acting as a preferential substrate. Once all the βCAR was consumed by reaction with the oxygen reactive species, the generation of mono- and dihydroperoxydized products from 20:4n-6 and their subsequent cleavage resumed.

Effects of ETYA and INDO. The addition of ETYA (10 μM) or INDO (10 μM) at the beginning of the irradiation period did not affect the various reaction parameters under control conditions (data not shown).

DISCUSSION

Nonenzymatic peroxidation of lipids results in a complex cascade of reactions (32,34–36). The *in vitro* model of lipid photoperoxidation described here illustrates this complexity. The kinetics of photoperoxidation of 20:4n-6 can be divided into three main steps: (i) monohydroperoxidation characterized by formation of HpETEs; these molecules can possess conjugated diene patterns; (ii) secondary oxidation characterized by formation of polyoxygenated products, including endoperoxides and DiHpETEs; the latter molecules can possess conjugated triene patterns; (iii) oxidative cleavage of the products of the two first steps into aldehydic molecules; these terminal molecules do not possess conjugated patterns and do react with thiobarbituric acid (TBA). The experimental results underline the intermediate appearance of monohydroperoxides and conjugated patterns during the cascade of reactions involved in lipid peroxidation. As early as in 1967, Esterbauer and Schauenstein (37) observed the transient formation of conjugated dienes during the oxidation of a methyl linoleate emulsion in water. In 1970, Tam and McCay (38) showed the transient formation of lipid peroxides during a kinetic study of radical peroxidation of liver microsomes. Terao and Matsushita (31), in a model of photoperoxidation of 20:4n-6 with methylene blue as photosensitizer, showed the early steps of the formation of HpETEs to be followed by secondary oxidation product formation such as DiHpETEs after a lag period of 4 h. These authors, as well as Chacon *et al.* (33), demonstrated the formation of eight positional isomers of HpETEs including the two nonconjugated isomers characteristic of peroxidation by singlet oxygen *via* ene addition. The observation of racemic mixtures of optical isomers of the isolated positional isomers of photoperoxidized HETEs is in agreement with Kuhn *et al.* (39) and is representative of nonenzymatic lipid peroxidation. The significant production of nonconjugated 14-HpETE and 6-HpETE in comparison with diene conjugated HpETEs produced at 90 min of irradiation indicates that during the first 90 min of irradiation the mechanism of monohydroperoxidation is purely or predominantly type II photoperoxidation (10,11,31,33,40). The lack of an effect of VitE in comparison with the total inhibition induced by βCAR during the first 60 min of irradiation confirms that nonradical peroxidation occurred during this period. The results emphasize the low singlet oxygen quencher effect of VitE in comparison with the important effect of βCAR. This is consistent with previous reports (18,41) which indicated that βCAR exhibits a physical quenching rate constant with singlet oxygen which is 50-fold greater than that of VitE. *In vivo*, the significant difference between the singlet oxygen quencher effects of βCAR and VitE may be compensated for by the plasma concentration. In plasma of healthy human population, Riemersma *et al.* (42) showed that the VitE concentration is more than 44-fold higher than the βCAR concentration. The lack of an effect of the free radical scavenger PROB also argues for the occurrence of nonradical monohydroperoxidation during the first 90 min of irradiation. The lack of TBARS production during this period confirms the predominance of a type II mechanism during the first 90 min (10,11).

In contrast, the reactions involved in the steps of secondary oxidation and cleavage of hydroperoxidized 20:4n-6 were predominantly type I photoperoxidation with radical mechanisms. Indeed, VitE and PROB inhibit both the production of conjugated trienes and the disappearance of conjugated dienes. Both molecules decrease the rate of disappearance of HpETEs after 90 min of irradiation. The significant production of TBARS after 120 min, indicative of the free radical cleavage of hydroperoxides, supports the argument of a significant involvement of type I photoperoxidation during this period (10,11). The involvment of singlet oxygen in secondary oxidation remains low under these experimental conditions because VitE and PROB did not inhibit the monohydroperoxidation of 20:4n-6 by singlet oxygen. Type 1 photoperoxidation, but no autoxidation, is predominantly involved in the fragmentation of peroxidized 20:4n-6 because when the irradiation is interrupted after the first 135 min of reaction, there is total inhibition of the oxidative cleavage of peroxidized 20:4n-6. The formation of conjugated dienes persists 60 min after the beginning of the disappearance of HpETEs, indicating that secondary oxidation products formed from HpETEs as endoperoxides and dihydroperoxides can enter into a peroxidation process yielding numerous molecules possessing conjugated diene patterns; these molecules could lead to a secondary generation of conjugated aldehydes (43).

Only the predominant oxidative reactions involved in the successive steps of 20:4n-6 photoperoxidation can be analyzed. The antioxidants used to screen oxidative mechanisms are not absolutely specific in their action. VitE, the main physiological free radical scavenger, is a low quencher of singlet oxygen, and βCAR, a powerful quencher of singlet oxygen, can act under certain conditions as a free radical scavenger (9,18,19).

VitE does not act as an inhibitor of the initiation step of autoxidation but scavenges chain-propagating radicals and inhibits the secondary oxidation and oxidative cleavage of monoperoxidized compounds (44–46). The reaction products of VitE with peroxyl radicals of methyl linoleate formed by autoxidation have recently been isolated and characterized (47).

The inhibitory effect of BW755C on each index of peroxidation after addition at 0 min or at 135 min of

irradiation demonstrates that in addition to its established free radical scavenging properties, this molecule can possess singlet oxygen quenching and/or scavenging effects. Janero *et al.* (48,49) demonstrated that BW755C is ten times more potent than VitE in protecting cardiac lipids against oxidative stress.

The lack of effects of ETYA on photoperoxidation of 20:4n-6 is representative of its mechanism of inhibition of eicosanoid metabolism. ETYA, a structural analogue of 20:4n-6, competitively inhibits both cyclooxygenase and lipoxygenase (24). Indeed, ETYA inhibits the release of prostacyclin but not the production of TBARS by cultured smooth muscle cells in response to the addition of 20:4n-6 (50). In the presence of VitE, the opposite result is obtained. The lack of a protective effect of INDO on photoperoxidation of 20:4n-6 is consistent with the lack of an antioxidant effect of this molecule against peroxidative damage of myocardial phospholipids (49). ETYA and INDO were used here as negative controls for a complete description of the limits and the selectivity of the model of 20:4n-6 photoperoxidation.

In our *in vitro* experiments, repeated sampling of the reaction mixtures permitted the detection of transient formation and subsequent degradation of monohydroperoxidized lipids and conjugated patterns. These observations point at "kinetic restrictions" which should be considered in the interpretation of *in vivo* studies of lipid peroxidation in which monohydroperoxidized lipids are measured by HPLC as the sole index and at a single time point (51–53). The coupling of the measurement of monohydroperoxidized lipids with the quantitative analysis of aldehydic products would be a valid index of the dynamics of the cascade of reactions involved in lipid peroxidation (54,55).

The case of BW755C, an inhibitor of both nonradical and radical peroxidation, underlines the limitations of the use of a photochemical process in ischemia-reperfusion studies. Indeed, for this type of oxidative injury, the involvement of "dark" generation of singlet oxygen has not been formally demonstrated.

Antioxidants are promising therapeutic agents for numerous diseases involving oxidative stress (56,57). The combination of singlet oxygen quenching and free radical scavenging properties could be avantageous for specific diseases in which singlet oxygen intervention is involved. The *in vitro* model of lipid photoperoxidation described here provides a simple and reproducible method for the preliminary screening of scavengers of reactive oxygen species of therapeutic interest.

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Eicosapentaenoic Acid at Hypotriglyceridemic Dose Enhances the Hepatic Antioxidant Defense in Mice

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The effect of oral administration of purified (95%) eicosapentaenoic acid on serum lipids, hepatic peroxisomal enzymes, antioxidant enzymes and lipid peroxidation was compared with that of palmitic acid fed mice and corresponding controls. After 10 d, a dose of 1000 mg eicosapentaenoic acid per day/kg body weight lowered serum triglycerides by 45%, while no significant change in serum cholesterol level was noted in comparison to palmitic acid fed mice and controls. Hepatic acyl-CoA oxidase and catalase activities increased by 50% and 30%, respectively, in the eicosapentaenoic acid fed group. In addition, the hepatic reduced glutathione content and the activities of glutathione transferase, glutathione peroxidase and glutathione reductase, increased significantly during eicosapentaenoic acid treatment. The levels of hepatic lipid peroxides were lower after eicosapentaenoic acid feeding, while no significant change was noted in the palmitic acid fed mice when compared to the controls. Taken together, the present data demonstrate for the first time that at hypolipidemic doses eicosapentaenoic acid feeding i) enhances the hepatic antioxidant defense, and ii) does not cause a significant differential induction of the two peroxisomal enzymes, acyl-CoA oxidase and catalase, as was noted after administration of hypolipidemic peroxisome proliferating compounds, such as clofibrate in rodents.
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Excess of lipids in the blood is considered to accelerate the development of arteriosclerosis and is a risk factor in myocardial infarction. Accordingly, reduction of high blood lipid levels by diet or by drugs is used as a preventive measure in people at risk (1,2).

Hypolipidemic drugs such as clofibrate, benzafibrate and nafenopin are effective in lowering serum lipids in various dyslipidemic disorders in human subjects (1,2) and experimental animals (3). However, at hypolipidemic doses, these compounds markedly induce peroxisome proliferation in rodents (3-5). This can lead to an increase in hydrogen peroxide generation due to the concomitant increase in peroxisomal β-oxidation which is an undesirable effect of the compounds as it may create cellular oxidative stress. In addition, these drugs have been shown to compromise the activities of the glutathione requiring detoxification enzymes, such as glutathione transferase and glutathione peroxidase (3). It is, therefore, likely that the combined effect of these changes on the metabolism of hydrogen peroxide may directly or indirectly induce membrane lipid peroxidation and oxidative damage to DNA (4,5). Ultimately, such changes are thought to be the cause for the increased incidence of hepatic neoplasia in rodents treated with hypolipidemic peroxisome proliferator compounds (6-8). For this reason there is a need to look for new hypolipidemic compounds with minimal effects on the peroxisomal H_2O_2 metabolizing enzymes and other antioxidant defenses in the liver.

Recently, there has been a growing interest in the hypolipidemic effect of the polyunsaturated fatty acids of fish oil origin and especially eicosapentaenoic acid (EPA, 20:5) (9-11). Furthermore, these fatty acids have been shown to inhibit the growth of colonic tumors in experimental animals (12) and are implicated to exert an inhibitory effect on tumor growth (13). In view of these findings, there is little or no work done on the effect of EPA on peroxisomal H_2O_2 metabolism and other cellular antioxidant defense mechanisms.

Based on these facts, it was of particular interest to investigate the effects of administering purified (95%) EPA on the activities of acyl-CoA oxidase and catalase, the two peroxisomal enzymes involved in the production and decomposition of H_2O_2, the levels of glutathione, and the glutathione requiring detoxification enzymes in mouse liver. The impact of these changes on hepatic lipid peroxidation will be discussed.

MATERIALS AND METHODS

Chemicals. Ethyl EPA (95% purity) was obtained from Norsk Hydro AS, Research Centre (Porsgrunn, Norway). Palmitic acid and glutathione reductase were purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals used were of analytical grade and were obtained from common commercial sources.

Animals and treatments. Male OF1 mice were purchased from IFFA Credo (Lyon, France). Two-week-old mice weighing 25-30 g were housed in groups of five in metal wire cages in a room maintained at a constant temperature of 20 ± 3°C. The animals were acclimated for one week under these conditions at 12-h light-dark cycles before the start of the experiment.

Stock suspensions (20% wt/vol) of EPA and palmitic acid (16:0) were prepared in 0.5% sodium carboxymethyl cellulose (CMC). α-Tocopherol (0.5% wt/vol) was added to the stock suspensions to minimize autooxidation. The fatty acids were administered to the mice by gastric intubation in a volume of 0.125-0.150 mL (1000 mg/kg body weight) once a day for 10 d. The first group received EPA, while the second group was fed 16:0 in an attempt to minimize the discrepancy in caloric intake with the first group; the third group was given an equal volume of the vehicle (CMC) used to suspend the fatty acids and are designated controls. While the animals were administered the selected compounds, they were fed a commercial pelleted diet and had free access to food and water.

On the 11th day, overnight fasted mice were lightly anesthetized and cardiac puncture was performed to obtain blood samples in heparinized tubes. The livers were removed immediately, weighed and then chilled on ice.

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Abbreviations: CMC, sodium carboxymethyl cellulose; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid (20:5n-3); GSH, reduced glutathione; HEPES, *N*-[2-hydroxyethyl]piperazine *N'*-[2-ethanesulfonic acid]; HPLC, high-performance liquid chromatography; TBARS, thiobarbituric acid reactive substances.

Plasma was prepared from the whole blood samples by centrifugation at 1000 × *g* for 10 min.

Preparation of tissue homogenates. The livers from individual mice were homogenized in ice-cold sucrose medium, containing 0.25 sucrose in 10mM *N*-[2-hydroxyethyl]piperazine *N'*-[2-ethanesulfonic acid] (HEPES) buffer and 1mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, using a Potter-Elvehjem homogenizer at 720 rev/min and two strokes of a loosely fitting Teflon pestle. The resulting total homogenates were then centrifuged at 600 × *g* for 10 min and the postnuclear fraction was used for the enzyme assays. Liver homogenate (10%) in 1.15% KCl and in 5% sulfosalicylic acid were prepared for the determination of lipid peroxidation and glutathione, respectively. All procedures were performed at 0–4°C. The various liver fractions were stored at −80°C until analyzed.

Enzyme assays and other analytical methods. The activity of peroxisomal acyl-CoA oxidase was measured as previously described by Small *et al.* (14). Catalase activity was determined by spectrophotometry at 240 nm by the method of Lück (15). The activity of glutathione peroxidase was measured as described by Flohé and Günzler (16) with *t*-butyl hydroperoxide as substrate. Glutathione transferase activity was measured according the method of Habig *et al.* (17) using 1-chloro-2,4-dinitrobenzene as a substrate, while the activity of glutathione reductase was measured by the method of Eklöw *et al.* (18). The levels of glutathione were quantitated by reverse-phase high-performance liquid chromatography (HPLC) and fluorescence detection as described by Svardal *et al.* (19). Briefly, 10% liver homogenate in 5% sulfosalicylic acid was centrifuged at 600 × *g* for 5 min, and the resultant supernatant was derivatized with the fluorescent reagent monobromobimane. The glutathione-bimane adduct was then separated and quantitated by HPLC and fluorometry. Lipid peroxidation was measured by using the thiobarbituric acid assay described by Ohkawa *et al.* (20).

Protein was assayed with the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA).

Lipid analyses were carried out using the monotest cholesterol enzymatic kit (Boehringer, Mannheim, Germany) and the Biopak triglyceride kit (Biotrol, Paris, France).

Statistical analysis and presentation of results. Data are presented as means ± SD. Five animals were used in each group. One-way analysis of variance was used to determine significant differences among the different groups. Where appropriate, the Fisher's Least Square Difference was used to determine the significance of differences between the means at the 95% confidence level.

RESULTS AND DISCUSSION

One of our recent studies had shown that EPA at a dose of 1000 mg/kg body weight/day lowers serum triglycerides in rats by about 40%. This effect was reached by the 10th day with no further decline in serum triglycerides after 15 d of treatment (N. Willumsen, J. Skorve, S. Hexeberg and R.K. Berge, manuscript submitted for publication). Based on these findings, similar doses of EPA and 16:0 were administered to mice for 10 d in the present study.

All mice treated with EPA and 16:0 gained weight at the same rate as did controls (data not shown). The amount of food consumed was similar in the different treatment groups indicating that the fatty acids administered had minimal effect on appetite at the doses given.

In comparison to the controls, significant changes were noted in liver weight, liver/body weight ratio and the hepatic protein concentration per gram liver in EPA treated mice (Table 1). As shown in Figure 1, EPA feeding caused a decrease in serum triglycerides by 45%, while there was no such reduction in the 16:0 fed mice as compared to the controls. This is in agreement with the previously documented hypotriglyceridemic effect of EPA in rats (21). In addition, the serum cholesterol level was little affected by either EPA or 16:0 feeding (Fig. 2). As illustrated in Table 2, EPA treatment caused an increase in the hepatic peroxisomal acyl-CoA oxidase by 50% as

TABLE 1

Effects of Eicosapentaenoic Acid (EPA) and Palmitic Acid (16:0) on Mouse Liver Weight, Liver/Body Weight and Liver Protein Concentration After Ten Days of Treatment[a]

Type of treatment	Liver weight (gram)	Liver/body weight[b]	Liver protein (mg/g liver)
Control	1.71 ± 0.06	0.052 ± 0.003	119 ± 11
16:0	1.73 ± 0.09	0.054 ± 0.004	121 ± 10
EPA	1.87 ± 0.19[c]	0.061 ± 0.002[d]	132 ± 9[d]

[a] All values are mean ± SD for five animals.

[b] Gram liver/gram body weight.

[c] Statistically significant different means from the controls at 95% confidence level.

[d] Statistically significant different means from both the controls and 16:0 fed mice at 95% confidence level.

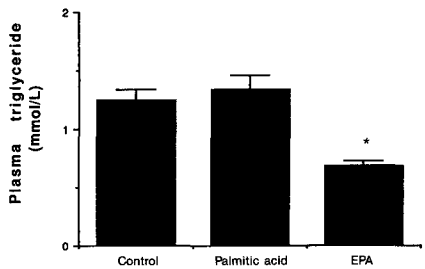

FIG. 1. Changes in plasma triglycerides in mice treated with eicosapentaenoic acid (EPA), palmitic acid (16:0) and the controls. *Statistically significant at 95% confidence level as compared to both the controls and 16:0 fed mice.

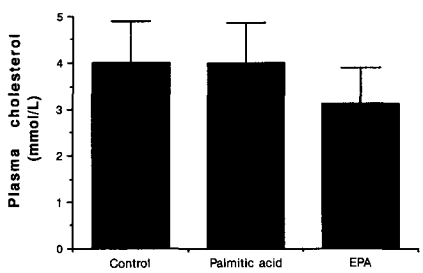

FIG. 2. Plasma cholesterol levels in mice treated with eicosapentaenoic acid (EPA) and palmitic acid (16:0) *vs.* controls.

TABLE 2

Changes in Hepatic Enzyme Activities in Mice Treated with Eicosapentaenoic Acid (EPA) and Palmitic Acid (16:0) After Ten Days of Treatment[a]

Enzyme activity	Control	16:0	EPA
Fatty acyl-CoA oxidase (μmol/min/g liver)	1.22 ± 0.09	1.34 ± 0.06	1.82 ± 0.06[b]
Catalase (mmol/min/g liver)	22.6 ± 2.4	22.9 ± 2.0	29.4 ± 1.8[b]
Glutathione peroxidase (μmol/min/g liver)	38.8 ± 1.8	34.6 ± 2.3	49.3 ± 1.6[b]
Glutathione transferase (μmol/min/g liver)	171 ± 16	169 ± 20	222 ± 16[b]
Glutathione reductase (μmol/min/g liver)	15.1 ± 2.1	12.3 ± 2.6	19.4 ± 3.4[b]

[a]All values are means ± SD of five animals.

[b]Statistically significant different means from both the controls and 16:0 fed mice at 95% confidence level.

compared to 16:0 fed mice and corresponding controls. This is in marked contrast to the 5- to 20-fold induction of the enzyme activity seen after administration of hypolipidemic doses of clofibrate in rodents (8,22). As peroxisomal oxidation of fatty acid generates H_2O_2, induction of acyl-CoA oxidase, the first key enzyme in the chain, is likely to be accompanied by increased production of H_2O_2. Furthermore, the activity of catalase, the main peroxisomal enzyme that degrades H_2O_2, was shown not to be induced proportionally after clofibrate treatment (8). It seems reasonable that the greater the difference in the extent of induction between acyl-CoA oxidase and catalase the more the imbalance would be in cellular H_2O_2 metabolism. Increased generation of H_2O_2 is detrimental to cellular function and DNA and is thought to be the underlying mechanism of the induction of hepatic neoplasia in rodents treated with peroxisome proliferating compounds (8,23). However, as illustrated in Table 2, the activity of catalase was increased by 30% in the EPA treated group when compared to the controls. Thus, the ratio of induction of acyl-CoA oxidase to catalase was about 1.2 in mice treated with EPA. This is far less than the ratio of about 5 shown with clofibrate treatment (8).

Along with the imbalance in peroxisomal H_2O_2 metabolizing enzyme activities, the levels of hepatic glutathione requiring detoxification enzymes are compromised by hypolipidemic drugs (3). In contrast to these findings, our present study shows that the treatment of mice with EPA enhances the activities of glutathione peroxidase, glutathione transferase and glutathione reductase by 27–30% when compared to both the 16:0 fed mice and the control group (Table 2). Furthermore, the level of reduced glutathione was noted to increase in the EPA fed group (Table 3). It is well known that glutathione peroxidase scavenges H_2O_2 and lipid peroxides (24). Hence, an increase in the activity of the enzyme may cause the modulation of H_2O_2 and of lipid peroxide metabolic processes in the liver. Unlike the hypolipidemic compounds of the clofibrate type, EPA feeding enhances the antioxidant defense in the liver of mice thereby increasing the capacity of the liver to challenge the damages inflicted by reactive oxygen species and other xenobiotic toxic metabolites.

TABLE 3

Changes in Hepatic Reduced Glutathione (GSH) and Lipid Peroxide Levels Measured as Thiobarbituric Acid Reactive Substance (TBARS) in Mice Treated with Eicosapentaenoic Acid (EPA) and Palmitic Acid (16:0) After Treatment for Ten Days[a]

Treatment group	GSH (μmol/g liver)	TBARS (nmol/g liver)
Control	5.4 ± 0.8	26.5 ± 1.5
16:0	5.7 ± 0.9	26.9 ± 1.4
EPA	8.7 ± 0.8[b]	20.5 ± 1.6[b]

[a]All values shown are means ± SD for five animals.

[b]Statistically significant different means from both the controls and 16:0 fed mice at 95% confidence level.

Reddy *et al.* (12) demonstrated that the incidence of colonic cancer is lower in rodents fed ω3 than ω6 fatty acids. Furthermore, EPA has been shown to have anticachexia and antitumor growth effects in rodents (13). Based on the present data, whether these effects can be attributed to enhanced antioxidant status by EPA has to be considered. The mechanism by which EPA enhances the antioxidant defense in the liver needs to be further investigated. Preliminary studies seem to suggest that EPA feeding diminishes the protein catabolic rate rather than the induction of their *de novo* synthesis (13).

The administration of EPA could lead to a corresponding increase in polyunsaturation of membrane lipids and thus could render cell membranes to be more susceptible for attack by reactive oxygen species. Contrary to this expectation membrane, lipid peroxidation as measured by thiobarbituric acid reactive substances (TBARS) assay was lower in the EPA than in the 16:0 fed mice and controls (Table 3). Although measurement of lipid peroxidation by this method is crude and controversial, the above finding suggests that both the increase in glutathione content and the activities of the glutathione requiring enzymes after EPA feeding (Table 3) may have protected membrane lipids against peroxidation. Increased glutathione reductase activity (Table 2) and effective reconversion of oxidized glutathione to its reduced form is one possible mechanism by which EPA enhances the reduced glutathione content.

In conclusion, the present data show for the first time that at hypotriglyceridemic doses of EPA the ratio of induction of acyl-CoA oxidase to catalase, the two main peroxisomal enzymes involved in the metabolism of hydrogen peroxide, is far less than the ratio of induction noted after the administration of clofibrate (8). Furthermore, EPA feeding enhances the levels of hepatic glutathione and its associated enzymes and thus improves the hepatic antioxidant defense in mice and thereby decreases membrane lipid peroxidation.

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Zinc Deficiency in the Rat Alters the Lipid Composition of the Erythrocyte Membrane Triton Shell

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The effect of dietary zinc deficiency on the lipid composition of the erythrocyte membrane Triton shell was determined. Weanling male Wistar rats were fed an egg white-based diet containing <1.0 mg Zn/kg diet *ad libitum*. Control rats were either pair-fed or *ad libitum*-fed the basal diet supplemented with 100 mg Zn/kg diet. A Zn refed group was fed the −Zn diet until day 18 and then pair-fed the +Zn diet until day 21. Dietary Zn deficiency caused an increased cholesterol/phospholipid ratio in Triton shells compared to those from pair-fed controls. Zn deficiency caused a decreased double bond index of fatty acids in phosphatidylinositol (PI) and phosphatidylcholine (PC); there was a decreased proportion of 18:2n-6 and 22:4n-6 in PC and 20:4n-6 in PI as compared to that found in pair-fed controls. All glycerophospholipids that were retained in the shell had a lower double bond index and increased content of 16:0 and/or 18:0 relative to the phospholipid in the intact membrane.
Lipids 27, 972-977 (1992).

The erythrocyte membrane from zinc-deficient rats has been used as a model system to test whether there is a physiological role of zinc in the structure and function of membranes (1-3). An alteration in the composition and/or the organization of the lipid domains of the membrane may be precipitated by dietary Zn deficiency. Erythrocytes from Zn-deficient rats are more susceptible to hemolysis induced by the anionic detergents sodium dodecyl sulfate and sodium dodecyl *n*-sarcosine, compared to those from pair-fed, Zn-adequate controls (4). Melittin, a protein which inserts itself into lipid bilayers and disrupts the permeability barrier of membranes (5,6), has increased hemolytic activity in erythrocyte membranes from Zn-deficient rats (4); on the other hand, dimethylsulfoxide, a solvent which perturbs the organization of lipid bilayers (7,8), has decreased hemolytic activity (4). The fat content (9) and fat type (10) of the diet have been shown to modify the increased osmotic fragility of erythrocytes from Zn-deficient rats, and Zn deficiency has been shown to exacerbate the effect of vitamin E deficiency on the peroxidative fragility of erythrocyte membranes (11).

Recent studies have suggested that dietary Zn deficiency does not have a dramatic effect on the fatty acid composition of the major phospholipids of erythrocyte membranes compared to those from pair-fed controls (12, 13). However, diminished levels of phosphatidylserine (PS), a phospholipid located on the inner surface of the mammalian erythrocyte membrane, suggests an altered asymmetry of erythrocyte membrane lipids in Zn deficiency (13). Mammalian erythrocyte membranes exhibit lipid asymmetry in terms of inner surface *vs.* outer surface steady-state distribution of individual phospholipids (14, 15) and in terms of lipid association with integral (16,17) and peripheral (18,19) membrane proteins. The lipid composition of asymmetric zones in the erythrocyte membranes has been probed by at least three methods—treatment of the outer membrane surface with phospholipases (20,21); release of protein and lipid containing microvesicles from the membrane (22,3); and selective solubilization of some lipids and proteins from the membrane (24-26). The present experiments were performed to examine the effect of dietary Zn deficiency on the lipid composition of the erythrocyte membrane Triton shell, *i.e.,* the lipids that remain associated with the membrane skeletal proteins in the presence of detergent.

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Abbreviations: aGPE, alkenylacyl glycerophosphoethanolamine; Chol, cholesterol; DBI, double bond index; dGPE, diacyl glycerophosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; −Zn, zinc-deficient rats; +ZnAL, zinc-adequate *ad libitum*-fed rats; +ZnPF, zinc-adequate pair-fed rats; −ZnRF, zinc-refed rats.

MATERIALS AND METHODS

Male weanling Wistar rats (45-58 g) were divided into zinc-deficient (−Zn), zinc-refed (−ZnRF), zinc-adequate, pair-fed (+ZnPF) and zinc-adequate *ad libitum* (+ZnAL) treatment groups and were fed the appropriate diet for three weeks. The basal diet (−Zn) contained <1 ppm Zn and was based on egg white, glucose and corn oil (27). Control diets (+ZnAL, +ZnPF) were supplemented to contain 100 μg Zn as $ZnCO_3$/g diet. The −ZnRF group was fed the basal diet *ad libitum* for 18 d and then pair-fed the Zn supplemented diet on days 19 to 21. Feed was removed from all rats 12 h prior to sacrifice. After light anesthesia with diethyl ether, the rats were killed by decapitation and trunk blood was collected in heparinized tubes. Plasma Zn concentration was determined by atomic absorption spectrophotometry (27).

Preparation of the erythrocyte membrane Triton shell. Erythrocyte ghosts were prepared according to the method described by Avery and Bettger (28). Triton shells were prepared according to the method of Yu *et al.* (24). One mL of packed ghosts was aliquoted to a 10-mL polycarbonate centrifuge tube and 7 mL of 1% (vol/vol) Triton X-100, 100 μM phenylmethysulfonylfluoride, 1 μM leupeptin and 1 μM pepstatin, in 5.0 mM sodium phosphate, pH 8.0 at 4°C, was added. The white ghost pellet immediately dispersed and the solution clarified. This solution was incubated on ice for 30 min without further agitation and then centrifuged at 4°C for 30 min at 28,000 × *g*. Careful removal of the supernatant fluid left a clear, Triton shell pellet (approximately 200-300 μL) which was frozen at −20°C until analyzed.

Biochemical analyses. Frozen pellets of Triton shells were thawed and then resuspended in 500 μL of 5 mM sodium phosphate, pH 8.0. To eliminate clumping, the shell suspension was sonicated at 60 mW power for 10 s on a 30% duty cycle on a Branson (Danbury, CT) model 300 sonicator. The resulting clear, apparently homogeneous solution was then assayed for protein, total cholesterol, phospholipid phosphorus and the fatty acid composition of individual phospholipid classes as previously

described for the analysis of erythrocyte ghosts (13). Freshly prepared Triton shells were analyzed for protein and glycoproteins by the method of Fairbanks *et al.* (29).

Statistics. Data were analyzed by a general linear modelling procedure followed by Tukey's studentized range test and a least square means analysis comparing −Zn to −ZnRF and +ZnPF groups. All data were examined to determine whether they represented a normally distributed population by calculation of the Shapiro-Wilk statistic on the residuals. Data were transformed prior to analysis, if necessary, to obtain a normal distribution (30).

RESULTS

At the end of the experiment the rats had the following weight gains (g/3 wk): −Zn 4.3 ± 0.7, −ZnRF 13.3 ± 1.4, +ZnPF 23.4 ± 1.7 and +ZnAL 130.3 ± 2.2; and plasma Zn concentrations (mg/L): −Zn 0.35 ± 0.03, −ZnRF 1.30 ± 0.05, +ZnPF 1.30 ± 0.06 and +ZnAL 1.64 ± 0.07. There were no mortalities associated with any of the dietary treatments.

Erythrocyte membrane Triton shells from −Zn rats had significantly less phospholipid per mg protein than those from +ZnAL rats and had a higher cholesterol/phospholipid ratio than both +ZnPF and +ZnAL rats. Cholesterol/mg protein, the phosphatidylcholine (PC)/sphingomyelin (SPH) molar ratio, and the unsaturation index of phospholipid fatty acids were not affected significantly by the dietary treatment (Table 1). Similarly, the mole percentage of phospholipids in the shell was not affected significantly by dietary treatment (Table 2). The protein composition of rat erythrocyte membrane Triton shells was virtually identical to that illustrated by Kunimoto *et al.* (32); in the Triton shells, most of the Band 3 had been removed and there was no remaining periodic acid-Schiff reagent-staining glycoprotein. There was no effect of dietary Zn deficiency on the protein composition of the shell as measured by densitometry of the stained gels (data not shown).

The Triton shell PC from −Zn rats contained significantly more saturated and less mono- and polyunsaturated fatty acids than +ZnPF controls. The feed restriction associated with dietary Zn deficiency resulted in less 20:4n-6, 22:4n-6 and 22:5n-6, and in more 18:2n-6 than found in +ZnAL rats (Table 3). In response to decreased

TABLE 2

Effect of Dietary Zn Deficiency on the Mass (mol%) of Phospholipids in the Erythrocyte Membrane Triton Shell

Phospholipid	Treatment group[a,b] −Zn	−ZnRF	+ZnPF	+ZnAL	Standard error[c]
PC	50.8	50.4	52.3	54.0	1.3
SPH	41.2	41.4	38.5	36.8	1.9
aGPE[d,e]	2.1	2.2	2.4	2.4	0.3
dGPE[f]	2.4	2.5	2.8	2.8	0.2
PS	17	1.8	2.2	2.3	0.2
PI	1.8	1.7	1.9	1.7	0.1

[a] Mean (n = 6, −ZnRF n = 3). Data are expressed as the % of the phospholipids quantitated. Abbreviations as in Table 1.

[b] Values for a given phospholipid are not significantly different ($P > 0.05$) by Tukey's test.

[c] Pooled standard error.

[d] aGPE, alkenylacyl glycerophosphoethanolamine.

[e] May contain small amounts of alkylacyl glycerophosphoethanolamine (ref. 31).

[f] dGPE, diacyl glycerophosphoethanolamine.

TABLE 1

The Effect of Dietary Zn Deficiency on the Phospholipid, Cholesterol and Fatty Acid Unsaturation Index in the Erythrocyte Membrane Triton Shells

Index	Treatment group[a] −Zn	−ZnRF	+ZnPF	+ZnAL	Standard error[b]
PL/mg P[c]	0.55[d]	0.59[d,e]	0.57[d,e]	0.64[e]	0.01
Chol/mg P[f]	0.72	0.72	0.68	0.77	0.01
Chol/PL[g]	1.30[h]	1.22	1.20	1.21	0.02
PC/SPH[i]	1.32	1.40	1.44	1.53	0.07
UI[j]	1.25	1.39	1.47	1.32	0.12

[a] Mean (n = 7; PC/SPH n = 7, +ZnAL n = 6, UI n = 6, −ZnRF n = 3). −Zn, zinc-deficient rats; −ZnRF, zinc-refed rats; +ZnPF, zinc-adequate, pair-fed rats; +ZnAL, zinc-adequate, *ad libitum*-fed rats.

[b] Pooled standard error.

[c] μmol Phospholipid (PL)/mg protein.

[d,e] Means that do not share a common superscript letter are significantly different by Tukey's test ($P < 0.05$).

[f] μmol Cholesterol (Chol)/mg protein.

[g] Cholesterol/phospholipid molar ratio.

[h] Values having a superscript letter h are significantly different than +ZnPF control by a least square means analysis ($P < 0.05$).

[i] Phosphatidylcholine (PC)/sphingomyelin (SPH) molar ratio.

[j] UI, unsaturation index (average number of double bonds) of the fatty acids from the phospholipids which were analyzed.

TABLE 3

Effect of Dietary Zn Deficiency on the Fatty Acid Composition (mol%) of Phosphatidylcholine in the Erythrocyte Membrane Triton Shell

Fatty acid	Treatment group[a] −Zn	−ZnRF	+ZnPF	+ZnAL	Standard error[b]
Saturated	78.4[c,d]	76.2[d,e]	74.5[d,e]	75.9[e]	0.4
16:0	64.2	61.7	61.9	62.5	0.4
18:0	13.5[c,d,e]	13.7[d]	11.8[f]	12.5[e,f]	0.1
Monounsaturated	7.2[c,d]	7.8[d,e]	9.0[f]	8.8[e,f]	0.1
Polyunsaturated	14.4[c]	16.0	16.5	15.3	0.3
Total n-6	13.9[c]	15.4	16.0	14.8	0.3
18:2n-6	10.8[c,d]	11.9[d]	12.4[d]	10.2[e]	0.3
20:4n-6	2.7[d]	3.1[d]	3.2[d]	4.0[e]	0.1
22:4n-6	0.10[c,d]	0.10[d]	0.13[d]	0.19[e]	0.004
22:5n-6	0.1[d]	0.1[d]	0.1[d]	0.3[e]	0.01
Total n-3	0.6	0.6	0.5	0.5	0.02
22:5n-3	0.1	0.1	0.1	0.1	0.004
22:6n-3	0.5	0.5	0.4	0.4	0.02
DBI[g]	0.44[c,d]	0.48[d,e]	0.51[e]	0.51[d,e]	0.01

[a] Mean (n = 7, +ZnAL n = 6). Abbreviations as in Table 1.

[b] Pooled standard error.

[c] Values having a superscript letter c are significantly different than +ZnPF by a least square means analysis ($P < 0.05$).

[d,e,f] Means that do not share a common superscript letter are significantly different by Tukey's test ($P < 0.05$).

[g] Double bond index.

food intake, SPH contained more saturated and less mono- and polyunsaturated fatty acids than +ZnAL controls, leading to a decreased double bond index (DBI) (Table 4).

The fatty acid composition of alkenylacyl glycerophosphoethanolamine (aGPE) and diacyl glycerophosphoethanolamine (dGPE) is shown in Tables 5 and 6. In aGPE, dietary Zn deficiency resulted in lower 22:4 and 22:5n-6 as compared to the +ZnPF group. Reduced food intake resulted in lower 22:5n-3 and 22:6n-3 compared to +ZnAL controls. In dGPE, Zn deficiency caused a significant elevation in 16:0 and a reduction in 22:5n-6 compared to +ZnPF controls.

In erythrocyte Triton shell PC, feed restriction was associated with decreased 22:5n-6 and increased 22:5n-3 (Table 7). In phosphatidylinositol (PI), Zn deficiency caused increased 16:0 and decreased 20:4n-6 compared to the +ZnPF group. It also resulted in a decreased DBI (Table 8).

TABLE 4

The Effect of Dietary Zn Deficiency on the Fatty Acid Composition (mol%) of Sphingomyelin in the Erythrocyte Membrane Triton Shell

Fatty acid	Treatment group[a]				Standard error[b]
	−Zn	−ZnRF	+ZnPF	+ZnAL	
Saturated	68.0[c]	68.4[c]	67.4[c]	59.1[d]	0.5
16:0	21.0	20.4	22.4	17.9	0.7
24:0	34.5[c]	35.4[c]	32.1[c,d]	29.4[d]	0.5
Monounsaturated	19.9[c]	19.8[c]	21.2[c]	24.8[d]	0.3
24:1n-9	19.6[c]	19.5[c]	20.6[c]	24.2[d]	0.4
Polyunsaturated	12.1[c]	11.8[c]	11.4[c]	16.1[d]	0.2
18:2n-6	trace	0.1	0.1	0.1	0.001
24:2n-6	12.1[c]	11.7[c]	11.3[c]	16.0[d]	0.2
DBI[e]	0.4[c]	0.4[c]	0.4[c]	0.6[d]	0.01
Average carbon number	21.9	21.9	21.7	22.2	0.06

[a]Mean (n = 7). Abbreviations as in Table 1.

[b]Pooled standard error.

[c,d]Means that do not share a common superscript letter are significantly different by Tukey's test ($P < 0.05$).

[e]Double bond index.

TABLE 5

Effect of Dietary Zn Deficiency on the Fatty Acid Composition (mol%) of Alkenylacyl Glycerophosphoethanolamine in the Erythrocyte Membrane Triton Shell

Fatty acid	Treatment group[a]				Standard error[b]
	−Zn	−ZnRF	+ZnPF	+ZnAL	
Saturated	19.1[d,e]	20.5[d,e]	16.0[d]	24.9[e]	1.1
16:0	16.9[d,e]	18.7[d,e]	14.7[d]	22.9[e]	1.1
18:0	1.0	1.3	0.8	1.5	0.2
Monounsaturated	5.7	6.3	4.0	3.8	0.5
Polyunsaturated	75.2	73.3	80.0	71.3	1.2
Total n-6	54.9[c]	54.4	60.3	59.4	0.8
18:2n-6	3.0	3.1	2.8	2.6	0.3
20:4n-6	39.1[d,e]	38.1[d,e]	41.1[d]	34.5[e]	0.8
22:4n-6	10.7[c,d]	10.9[d]	13.6[e]	18.0[f]	0.2
22:5n-6	1.4[c,d]	1.9[d,e]	2.4[e]	4.2[f]	0.1
Total n-3	20.4[d]	18.9[d]	19.7[d]	11.9[e]	0.6
22:5n-3	11.1[d]	10.0[d]	10.7[d]	6.4[e]	0.4
22:6n-3	9.3[d]	8.9[d]	9.0[d]	5.5[e]	0.3
DBI[g]	3.3	3.2	3.5	3.1	0.006

[a]Mean (−Zn n = 6, −ZnRF n = 5, +ZnPF n = 6, +ZnAL n = 7). Abbreviations as in Table 1.

[b]Pooled standard error.

[c]Values having a superscript letter c are significantly different than +ZnPF by a least square means analysis ($P < 0.05$).

[d,e,f]Means that do not share a common superscript letter are significantly different by Tukey's test ($P < 0.05$).

[g]Double bond index.

TABLE 6

Effect of Dietary Zn Deficiency on the Fatty Acid Composition (mol%) of Diacyl Glycerophosphoethanolamine in the Erythrocyte Membrane Triton Shell

Fatty acid[a]	Treatment group[b]				Standard error[c]
	−Zn	−ZnRF	+ZnPF	+ZnAL	
Saturated	46.4[d]	43.9	42.2	42.2	0.6
16:0	32.8[d]	30.3	29.3	30.0	0.5
18:0	12.3	12.4	12.0	11.4	0.3
Monounsaturated	19.0	18.3	18.8	20.8	0.7
Polyunsaturated	34.6	37.8	39.0	37.0	0.8
Total n-6	29.2[d]	31.3	33.2	33.4	0.4
18:2n-6	9.3	10.0	9.9	9.8	0.2
20:4n-6	17.2	18.6	20.1	19.3	0.5
22:4n-6	1.6[e]	1.6[e]	1.9[e,f]	2.4[f]	0.1
22:5n-6	0.3[d,e]	0.3[e]	0.5[e]	1.0[f]	0.04
Total n-3	5.4[e,f]	6.6[e]	5.8[e,f]	3.7[f]	0.3
22:5n-3	1.8[e,f]	2.2[f]	1.8[f]	1.3[e]	0.1
22:6n-3	3.6	4.3	4.0	2.4	0.2
DBI[g]	1.47	1.60	1.65	1.56	0.03

[a]May contain small amounts of fatty acids from alkylacyl glycerophosphoethanolamine (ref. 31).

[b]Mean (n = 7). Abbreviations as in Table 1.

[c]Pooled standard error.

[d]Values having a superscript letter d are significantly different than +ZnPF by a least square means analysis ($P < 0.05$).

[e,f]Means that do not share a common superscript letter are significantly different by Tukey's test ($P < 0.05$).

[g]Double bond index.

DISCUSSION

The Triton shell referred to in this paper is the erythrocyte membrane Triton shell as originally defined by Yu *et al.* (24), and subsequently described by several researchers in various biochemical and ultrastructural studies (23,25,33–39). Triton shells can be derived from intact red cells or from isolated erythrocyte membranes under a variety of conditions (25). The exact lipid and protein composition of the human erythrocyte Triton shell is thought to vary with pH, temperature, detergent concentration, time of extraction, ionic strength and the composition and structural arrangement of the proteins and lipids in the membrane (18,24,25,40). It has been stated succinctly by Niggli and Berger (18) that "co-isolation of components with the Triton X-100 insoluble cytoskeleton is at best an indication, but never a conclusive proof, for a specific interaction of proteins or lipids with the cytoskeleton. . . ." However, in the original report by Yu *et al.* (24), free ^{32}P-labelled phospholipid mixtures, when incubated with the Triton shells, did not show significant exchange with shell phospholipids, nor were they deposited on the shell

TABLE 7

Effect of Dietary Zn Deficiency on the Fatty Acid Composition (mol%) of Phosphatidylserine in the Erythrocyte Membrane Triton Shell

Fatty acid	Treatment group[a]				Standard error[b]
	−Zn	−ZnRF	+ZnPF	+ZnAL	
Saturated	47.8	50.1	48.2	49.5	1.8
16:0	14.7	16.0	13.9	15.0	1.1
18:0	31.7	32.4	31.9	33.0	1.1
Monounsaturated	5.2	7.0	6.6	6.9	0.7
Polyunsaturated	47.0	42.9	45.3	43.6	1.8
Total n-6	40.2	37.2	39.7	40.2	1.5
18:2n-6	3.7	3.9	3.4	3.2	0.1
20:4n-6	32.9	30.7	32.7	32.4	1.5
22:4n-6	1.6[c]	1.4[c]	1.7[c,d]	2.1[d]	0.1
22:5n-6	1.0[c]	0.9[c]	1.1[c]	2.1[d]	0.1
Total n-3	6.8[c]	5.7[c,d]	5.5[c,d]	3.4[d]	0.3
22:5n-3	1.5[c]	1.2[c]	1.2[c]	0.7[d]	0.1
22:6n-3	5.4[c]	4.4[c,d]	4.3[c,d]	2.7[d]	0.3
DBI[e]	2.0	1.8	1.9	1.8	0.1

[a]Mean (n = 7, −ZnRF n = 5). Abbreviations as in Table 1.

[b]Pooled standard error.

[c,d]Means that do not share a common superscript letter are significantly different by Tukey's test ($P < 0.05$).

[e]Double bond index.

TABLE 8

Effect of Dietary Zn Deficiency on the Fatty Acid Composition (mol%) of Phosphatidylinositol in the Erythrocyte Membrane Triton Shell

Fatty acid	Treatment group[a]				Standard error[b]
	−Zn	−ZnRF	+ZnPF	+ZnAL	
Saturated	69.2c,d,e	70.0[d]	62.7[e]	62.7[d,e]	0.9
16:0	32.8[c,d]	30.9[d,e]	28.3[d,e]	26.6[9e]	0.7
18:0	34.4	37.7	33.0	34.9	0.7
Monounsaturated	8.0	5.3	6.6	8.0	0.6
Polyunsaturated	22.8[c,d]	24.7[d]	30.7[e]	29.2[d,e]	0.7
Total n-6	20.6[c,d]	23.0[d,e]	28.5[f]	26.5[e,f]	0.4
18:2n-6	5.6	5.8	6.7	5.1	0.3
20:4n-6	13.6[c,d]	15.2[d,e]	19.8[f]	18.5[e,f]	0.5
22:4n-6	0.5[d]	0.8[d]	0.9[d,e]	1.5[e]	0.1
22:5n-6	0.2	0.3	0.3	0.4	0.1
Total n-3	2.2	1.7	2.2	1.3	0.2
22:5n-3	0.8	0.5	0.6	0.3	0.1
22:6n-3	1.4	1.3	1.4	0.9	0.2
DBI[g]	1.3[c,d]	1.4[d]	1.9[e]	1.9[d,e]	0.06

[a]Mean (n = 7). Abbreviations as in Table 1.

[b]Pooled standard error.

[c]Values having a superscript letter c are significantly different than +ZnPF by a least square means analysis ($P < 0.05$).

[d,e,f]Means that do not share a common superscript letter are significantly different by Tukey's test ($P < 0.05$).

[g]Double bond index.

structure. This suggests that the lipids of the shell are tightly associated with specific membrane proteins.

The lipid composition of the rat erythrocyte membrane Triton shell has not been described previously. The Triton shells used in the present study represent, for the most part, the same samples used for our previously reported (13) analysis of the effect of Zn deficiency on the lipid composition of the intact membrane. Samples were analyzed in parallel; this permitted us to make comparisons of shell lipid composition to intact membrane composition with considerable confidence. The phospholipid composition of the rat erythrocyte membrane Triton shell, when compared to the composition of the erythrocyte ghost (13), suggests that phospholipids are retained according to the following order: SPH >>> PC >> PI > dGPE > PS = aGPE. This is similar to the results of Yu *et al.* (24) for the human erythrocyte Triton shell which retains in the order: SPH > PC > phosphatidylethanolamine (PE) > PS. However, the final phospholipid profile is significantly different for the rat and human erythrocyte membrane Triton shell. The human shell has approximately 54–70% SPH, 20–25% PC and 10–17% PE. In contrast, the Triton shell from rat erythrocytes has more PC than SPH and less than 6% PE; a phenomenon which may partially reflect the differences in the ratio amounts of PC, SPH and PE in the membrane of the two species. The cholesterol/phospholipid ratio is elevated in the rat erythrocyte Triton shell by approximately 70% as compared to the intact ghost, whereas the data of Yu *et al.* (24) suggest that the cholesterol/phospholipid ratio is relatively constant between ghosts and shell in the human erythrocyte. The calculated cholesterol/SPH ratio remains relatively constant in the ghosts and shell in the rat. Assuming a 90% recovery of SPH, the minimum mass loss that can account for the change in SPH fatty acid composition in the shell *vs.* the intact membrane (13), the following recoveries can be estimated: protein 43%, cholesterol 51% and phospholipid 31%. Individual phospholipid recoveries can be estimated to be: PC 35%, SPH 90%, aGPE 5.8%, dGPE 9.2%, PS 5.1% and PI 11.8%. Kunimoto *et al.* (32), who prepared rat erythrocyte membrane Triton shells under conditions of high ionic strength, reported a 22% recovery of phospholipid and a similar phospholipid composition in the Triton shells.

Our studies with the rat erythrocyte Triton shell suggest that phospholipids are retained in Triton shells according to the head group, but also according to their fatty acid composition. For all phospholipids except SPH, the phospholipids retained in the shell have a lower DBI than those found in the intact membrane in our previous report (13); there is a substantial enrichment of 16:0 in PC and 16:0 and 18:0 in PS, aGPE, dGPE and PI retained in the Triton shell. Interestingly, PS, PI, aDPE and gDPE retain a higher proportion of 22:4n-6 compared to 20:4n-6 and 22:6n-3 when comparing ghost *vs.* shell phospholipid fatty acid composition (13). In contrast, the SPH retained in the shell is diminished in 16:0 and 18:0 fatty acids and enriched 22- and 24-carbon saturated and unsaturated fatty acids (13). The speculation of Sheetz (25) that SPH is retained because of its high proportion of saturated fatty acids could not be confirmed for the rat erythrocyte Triton shell where SPH containing long-chain fatty acids are preferentially retained relative to SPH containing 16:0 and 18:0. The structure of the sphingosine base that is part of retained SPH molcules may be a factor in its retention in the shell, as no other phospholipid associated with the shell is enriched in long-chain fatty acids (41). The composition of the lipids of the Triton shell, specifically cholesterol/PC, PC/SPH, unsaturation index and the DBI of individual phospholipid classes, suggests that the lipid domains are much less "fluid" than those of the intact

membrane. A similar phenomenon may occur in the Triton shells of stimulated and unstimulated rabbit and/or human platelets; there have been reports of enhanced recovery of palmitate-containing phospholipids relative to arachidonate-containing phospholipids in platelet Triton shells (42-44).

The effect of dietary Zn deficiency and the associated drop of voluntary food intake on the composition of the erythrocyte membrane Triton shell generally reflects the differences in the lipid composition found in the intact membrane (13), with some notable exceptions. Decreased feed intake causes a significant decrease in shell-associated phospholipid, as it does in intact membranes (13). The cholesterol/phospholipid ratio is only elevated in the shells of Zn-deficient rats; in intact membranes, all feed restricted groups have significantly elevated cholesterol/phospholipid ratios. Although there are no significant dietary effects on the profile of shell-associated phospholipids, it would appear that −Zn rats have shells that are depleted of PS, aGPE and dGPE (on a protein basis) compared to *ad libitum*-fed and, possibly, pair-fed controls. A similar trend exists in the intact erythrocyte membranes of −Zn and +ZnAL rats (13). In terms of fatty acid composition, the feed restriction associated with dietary Zn deficiency decreases the 22:4n-6 and 22:5n-6 in PC, aGPE, dGPE and PS and 24:2n-6 in SPH, and increases the n-3 polyunsaturated fatty acids in PS, aGPE and dGPE as it does in intact erythrocyte membranes (13). Similarly, the elevation in 18:2n-6 and the depression of 20:4n-6 in the shell-associated PC is an effect of feed restriction which stems from altered composition of the intact membrane (13). The biochemical and/or physiological basis for the carryover of diet-induced changes in the fatty acid composition of individual phospholipids into the phospholipids of the Triton shell is unknown.

There are two alterations attributable to dietary Zn deficiency (not to feed restriction) that are unique to shell-associated lipids and may be of some physiological significance. First, there is an elevated cholesterol/phospholipid ratio associated with the Triton shell of zinc-deficient rats. The effect appears to be attributable to decreased PS, aGPE, dGPE and, to a lesser extent, PC (Tables 1 and 2). The loss of these particular phospholipids may suggest a decreased binding affinity of these inner membrane bilayer-associated proteins for membrane proteins. Inner membrane surface PS and PE are bound, under some conditions, with specific cytoskeletal proteins such as spectrin (45,46) and protein 4.1 (47-49). The second change associated with zinc deficiency is a decrease in the 20:4n-6 concentration in PI in erythrocyte Triton shells. This may suggest that there is an alteration in the role of PI and, possibly, polyphosphoinositides in the membrane. Recent data suggests that arachidonoyl-containing PI and polyphosphoinositides may have a special role in the sensitivity of membranes to anesthetics (50), and polyphosphoinositides are thought to be critical for erythrocyte membrane integrity (51,52). Whether the decrease in shell-associated arachidonoyl-containing PI is a factor in the altered sensitivity to certain hemolysins (4), and/or is a factor in the reduced levels of arachidonoyl-containing polyphosphoinositides in erythrocyte membranes from zinc-deficient rats (53) warrants further investigation.

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Increased Globotriaosylceramide in Familial Dysautonomia

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Familial Dysautonomia (FD) is an autosomal recessive disease of unknown etiology, occurring primarily in Ashkenazi Jews. Patients are neurologically impaired, with deficits primarily in autonomic and sensory functions. The biochemical and genetic defects have remained elusive, precluding carrier detection and prenatal diagnosis. High-performance liquid chromatography data indicated up to a threefold increase in the neutral glycosphingolipid globotriaosylceramide in Dysautonomic fibroblasts and lymphoblasts. Total ganglioside values, measured by colorimetric, fluorometric or specific sodium borohydride incorporation, were decreased. Affected fibroblasts exhibited a range of pleomorphic phenotypes, such that the usual swirl-like confluent growth pattern of normal fibroblasts was distorted to varying degrees, suggesting abnormalities in the FD plasma membrane, possibly affecting cell-cell contacts. The glycosphingolipid increase could not be accounted for on the basis of markedly decreased α-galactosidase activity, as in Fabry's disease, where patients also display decreased autonomic function.
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Familial Dysautonomia (FD) is an autosomal recessive genetic disease of unknown etiology, involving deficits in autonomic, central and sensory functions (1). It is generally found within the Ashkenazi Jewish population, with an incidence of 1/3700 (2). Some clinical features include vasomotor instability, absence of fungiform papillae on the tongue, scoliosis, pulmonary complications, poor suckling, gastrointestinal problems, neurological impairments such as ataxia, absent or diminished deep tendon reflexes, impaired taste and temperature perception, reduced or absent overflow tears, peripheral nerve abnormalities, and a markedly reduced response to intradermal histamine (3). A defect in catecholamine metabolism was originally proposed, based on often decreased serum levels of dopamine-β-decarboxylase (4) and elevated urinary ratios of homovanillic acid to vanillylmandelic acid (dopamine and epinephrine metabolites, respectively) (5). The nerve growth factor receptor system, implicated for many years, has been shown by molecular biological techniques to be normal (6,7). Wrathall (8) demonstrated that the ability of fibroblasts to stimulate the outgrowth of neurites from newborn mouse sensory neurons is reduced when using FD cells or conditioned media derived therefrom. Complexing of all the nerve growth factor with antibody did not diminish the effect, implicating other unidentified factors.

Based on these observations and on the dramatic effects of exogenous gangliosides on neurite outgrowth (9–11), we began to examine glycosphingolipids (GSL) in FD. It is well known that glycoproteins and GSL cell surface components play major roles in cell differentiation, reception, recognition, myelination, synaptogenesis and neuronal transmission (12–14). Our goal was to quantitate the amount of the four major neutral GSL, namely GL_1 (glucocerebroside, GlcCer); GL_2 [lactosylceramide, Gal(β1-4)GlcCer]; GL_3 [globotriaosylceramide, Gal(α1-4)Gal(β1-4)GlcCer] and GL_4 [globotetraosylceramide, GalNac(β1-4)Gal(α1-4)gal(β1-4)GlcCer], and the total gangliosides in FD *vs.* those in normal fibroblasts, and to correlate any differences with microscopic changes associated with abnormal membrane GSL composition.

MATERIALS AND METHODS

Karyotyping. A single male proband and his father were used for chromosomal studies in the chromosome laboratory at the Hospital for Sick Children in Toronto. Chromosomes were prepared after the colcemid arrest of mitotic phytohemagglutinin-stimulated lymphocytes using G banding and high resolution banding procedures.

Micrography. Growth of FD and normal fibroblast cell lines was monitored by phase contrast microscopy; when confluent, cultures were photographed on T-MAX 100 (Kodak) film.

Tissue culture. Six FD fibroblast lines and one FD lymphoblast line from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ) were used: GM 00850A male; GM 02341 male; GM 02342 male; GM 02343 female; GM 04638 female; GM 04663 female; and GM 05041 male. Lymphoblasts from one local male patient (HSC 3654) were used as well. Two each of normal fibroblast and lymphoblast lines were obtained from the Hopsital for Sick Children cell bank. Fibroblasts were grown in α-MEM (Eagle's modified with Earle's salts, ref. 15) containing penicillin and streptomycin (Penicillin-Streptomycin, lyophilized, 10000 U/mL Penicillin G, 10000 μg/mL Streptomycin sulfate, Gibco, Toronto, ON, rehydrated to 20 mL, and used at a concentration of 1% in the growth medium), using 10% fetal calf serum. RPMI 1640 medium, containing 15% serum, and penicillin and streptomycin, was used for growing lymphoblasts. When serum-free medium was desirable, 1% HL-1 Supplement (Fisher, Toronto, Ontario, Canada) containing penicillin and streptomycin was added to maintain cell growth.

Neuritogenesis. Control and FD cells were grown to confluency in 24-well plates and seeded with human NUB-6C4A3 clone neuroblastoma cells. The induction of

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; FD, Familial Dysautonomia; GA_2, asialoGM_2, GalNAc(β1-4)Gal(β1-4)GlcCer; GL_1, GlcCer, glucocerebroside; GD_{1a}, NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)GlcCer; GD_{1b}, Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)GlcCer; GL_2, lactosylceramide, Gal(β1-4)GlcCer; GL_3, globotriaosylceramide, Gal(α1-4)Gal(β1-4)GlcCer; GL_4, globotetraosylceramide, GalNac(β1-4)Gal(α1-4)gal(β1-4)GlcCer; GM_1, Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)GlcCer; GM_2, GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)GlcCer; GM_3, NeuAc(α2-3)gal(β1-4)GlcCer); GSL, glycosphingolipids; GT_{1b}, NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)GlcCer; HPTLC, high-performance thin-layer chromatography; MG-3 or -4, NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcCer.

neurites with 10^{-6} M retinoic acid in NUB-6C4A3 cultures and characterization of this cell line have been reported (16).

Ganglioside extraction. Cells (1 mL of saline added to fresh or frozen pellet per T75 flask) were extracted with 10 vol of chloroform/methanol (1:2, vol/vol) with stirring for 1 h; then chloroform was added to change the chloroform/methanol ratio to 2:1 (vol/vol) and the extraction was continued for 30 min (17). KCl, 0.9%, in an amount equal to 1/5 of the total extraction volume was added (18) and after centrifugation the lower phase was washed with chloroform/methanol/0.9% KCl (3:48:47, vol/vol/vol). The combined upper phases were applied to a Sep-Pak C18 cartridge (Waters, Millipore Corp., Milford, MA) (17) pre-equilibrated in the same solvent, the cartridges were washed with 25 mL of water, and the gangliosides were eluted with 20 mL of methanol. Gangliosides were concentrated by rotoevaporation, taken up in a minimum volume of chloroform/methanol (1:1, vol/vol), sonicated, and run on high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) using chloroform/methanol/0.25% $CaCl_2$ (75:67.5:15, by vol). Each T75 flask of cells contained approximately 1 μg of sialic acid, or 4 μg of ganglioside. The HPTLC bands were difficult to see with a resorcinol spray (0.2 g resorcinol, 80 mL concentrated HCl, 0.1 mL of 0.1 M $CuSO_4$ in 100 mL total volume) since the 1-μg amount was associated with 3 to 10 bands (see below for AUF sensitivity). Gangliosides extracted from cells in several T75 flasks (where the equivalent amount of protein was 1 to 3 mg) were spotted per lane for detection. Ganglioside standards were obtained as follows: GM_3 and GT_{1b} were purchased from Sigma (Windsor, Ontario, Canada). GM_2 was purified in our laboratory from Tay-Sachs brain by Dr. Anton Novak. GM_1, GD_{1a} and GD_{1b} were generously donated by Dr. John Callahan (Hospital for Sick Children, Toronto, Ontario, Canada). The ganglioside nomenclature used in this paper is that described by Ledeen and Yu (19).

Neutral GSL determination. The neutral GSL (GL_1, GL_2, GL_3, and GL_4) are present in the lower phases of lipid extracts prepared as described above. The lower phases obtained from extracts of cells from 2 to 4 T75 flasks were dried under nitrogen, taken up in 1 mL of chloroform, and applied to 80 mg columns of silica gel, previously equilibrated with chloroform. The columns were washed with 2 mL of chloroform, and the neutral GSL eluted with 4 mL acetone/methanol (4:1, vol/vol) (20). The GSL were benzoylated and analyzed by high-performance liquid chromatography (HPLC), using monogalactosyldiglyceride as an internal standard, according to our standard procedure (21,22). In some cases, the underivatized GSL were dried and spotted directly onto thin-layer chromatography (TLC) plates (Sil G25, Macherey Nagel, Düren, Germany). Standard GSL were obtained as follows: GL_1 was purified in our laboratory from Gaucher spleen by Mrs. M. A. Skomorowski; GL_2 and GL_3 were purified in our laboratory as described previously (21); GL_4 was purchased from Matreya (Pleasant Gap, PA). The internal monogalactosyldiglyceride standard used in HPLC (21,22) was from Supelco (Oakville, ON).

Extraction of gangliosides and neutral GSL from media. Fifty mL of medium was centrifuged twice at 15,000 × *g* for 30 min to eliminate any extraneous cells, and then freeze-dried. Water (5 mL) was added, and gangliosides or GSL was extracted as above.

Quantitation of total cellular gangliosides. Two methods were used for this purpose. The colorimetric procedure is based on the reaction of sialic acid bound to ganglioside with resorcinol (19). Fibroblasts of one T75 flask contained approximately 1 μg of sialic acid corresponding to a reproducible AUF at 580 nm of 0.015 at 1 μg per mL. The fluorometric procedure depends on reaction with 3,5-diaminobenzoic acid (23). One μg of sialic acid reads 30 at maximum sensitivity on the Perkin-Elmer 650-40 fluorometer (Norwalk, CT).

Tritiation of gangliosides. Gangliosides were specifically labelled with NaB^3H_4 after periodate treatment (24).

Tritiation of GL_3. GL_3 was labelled by Dr. John Callahan according to the galactose oxidase procedure (25).

Loading cells with GL_3. Normal and FD fibroblasts were seeded onto P35 plates (Falcon 3001, Becton Dickinson and Co., Lincoln Park, NJ) and grown to confluency. [^{3}H]GL_3, 3.4×10^5 cpm, and 0.5 nmoles of cold carrier, were sonicated in 1 mL of α-MEM, without fetal calf serum (FCS), with penicillin and streptomycin added. The radioactive GL_3 medium was used to overlay the cells in place of the normal growth medium. After 24 h, the radioactive medium was replaced with normal medium containing 10% FCS. After another 24 h, fresh medium was reapplied. Ninety-six hours later, the amount of radioactive galactose liberated into the medium was measured. The percent hydrolysis was expressed as the radioactive galactose released into the medium divided by the sum of the radioactivity both in the released galactose and in the [^{3}H]GL_3 remaining in the cells (26). In other experiments, the [^{3}H]GL_3 was added to medium containing 10% FCS, and the percentage hydrolysis measured after 6 d with no media changes (26).

Debenzoylation and α-galactosidase digestion of GL_3. The benzoylated GL_3 fraction from the GSL standard mixture, from control cells, and the putative benzoylated GL_3 fraction from FD cells were collected as they emerged from the HPLC column. The fractions were debenzoylated by mild alkaline hydrolysis (27) after removing hexane/isopropanol with a stream of nitrogen. For this purpose, the dried benzoylated GSL was dissolved in 1 mL 0.6 N methanolic NaOH, plus 1 mL chloroform, and incubated at room temperature for 1 h. After addition of 1.7 mL of water and 3.4 mL of chloroform, the phases were mixed, and the lower phases were washed with methanol/water (1:1, vol/vol), and dried under nitrogen. One-half of the standard and of the FD debenzoylated GL_3 were digested to GL_2 using α-galactosidase (28) as follows: 500 μg of taurodeoxycholate was added to the dried GSL, and the mixture resuspended in 0.5 mL of 50 mM sodium citrate buffer, pH 4.5, containing 20 mM ethylenediaminetetraacetic acid (EDTA) and 1% bovine serum albumin (BSA). One unit of α-galactosidase (Sigma Chemical Co., St. Louis, MO) was added, and incubations were carried out overnight at 37°C. GL_2 and the remaining undigested GL_3 were extracted as above.

Enzyme studies. Cells were sonicated for ten seconds in phosphate-buffered saline, and assays for lysosomal hydrolases were performed for β-glucosidase, β-galactosidase, α-galactosidase, β-hexosaminidase, and α-neuraminidase using 4-methylumbelliferyl substrates (29), and natural substrates for β-galactocerebrosidase (30) and GM_1-β-galactosidase (31). Lignoceroyl acid oxidation, as a measure of peroxisomal integrity, was also determined (32).

RESULTS AND DISCUSSION

FD fibroblasts exhibited a normal karyotype. When grown on tissue culture plastic, FD fibroblasts attained confluency at a rate similar to that attained by normal fibroblasts but exhibited a range of pleomorphic phenotypes. The usual swirl-like confluent growth pattern of normal fibroblasts (Fig. 1A) was distorted to varying degrees in FD fibroblasts (Figs. 1B to D). FD fibroblasts ranged in phenotypes from the ones resembling the normal fibroblasts to enlarged, flattened cells overlapping in a random manner. This observation suggested possible aberrations in FD cell-cell interactions which could be clues to changes in plasma membrane cell surface components, *e.g.*, GSL.

The neuritogenic effects of cells and conditioned media on neuroblastoma cells were tested before we began the biochemical studies. As described in Materials and Methods, FD and control cells were grown to confluency and seeded with human NUB-6 C4A3 clone neuroblastoma cells. The cultures were induced with 10^{-6} M retinoic acid, required to induce neurite formation (21). No difference in the degree of neurite formation or in the proliferation of the neuroblastoma cells seeded on FD or control cells was found, nor was there any difference in these parameters when neuroblastoma cells were overlayed with conditioned medium from FD or control fibroblasts. These findings did not support the observations reported by Wrathall (8) according to which FD conditioned medium or FD cells inhibited the outgrowth of neurites from newborn mouse sensory neurons. Possibly mouse-human cocultures behave differently or perhaps our model which requires retinoic acid for the induction of neurites may not involve GSL at the site of interaction between retinoic acid and the cell membrane. Since human neuroblastomas represent a wide spectrum of peripheral nervous system types, other cell lines may have to be surveyed using this assay to develop a human bioassay model for inhibition which could be used to isolate the inhibitory factor(s).

HPLC determination of the four major neutral GSL (Table 1) revealed a significant increase in GL_3 in FD fibroblasts and lymphoblasts. Nervous tissue is generally not available for such studies. However there was a considerable range in values. The fibroblast FD values for GL_3 ranged from 1.98 to 24.31 nmoles/mg cell protein; the control fibroblast values ranged from 0.85 to 7.41 nmoles/mg cell protein. Cell lines with the greatest degrees

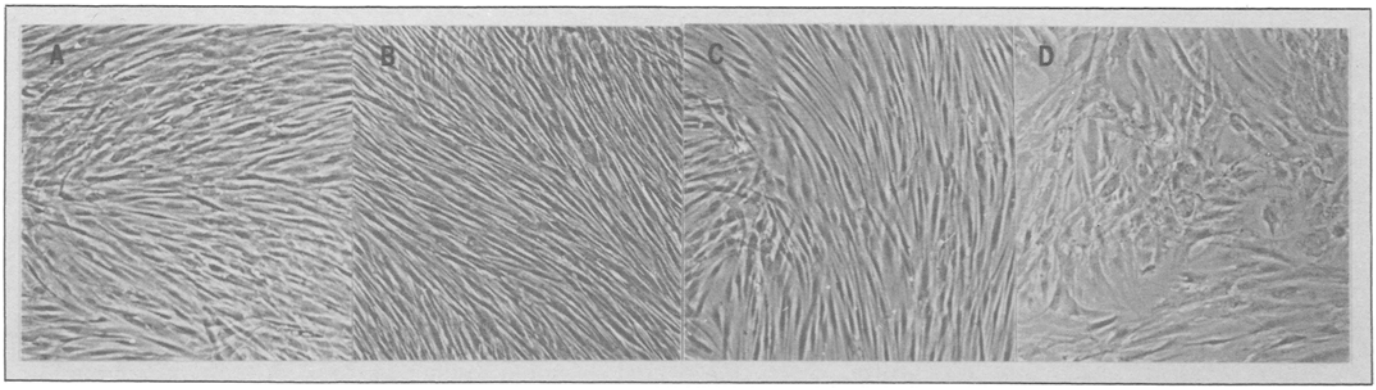

FIG. 1. Phase contrast microscopy of Familial Dysautonomia (FD) and normal fibroblasts. A: normal fibroblasts; B: FD line GM02343 (see Materials and Methods) displaying a mildly distorted phenotype; C: FD line GM02342, displaying a moderate degree of distortion; D: FD line GM00850, described as clinically affected, displaying a severe form of altered phenotype.

TABLE 1

Glycosphingolipid (GSL) Levels in Cells[a]

	Neutral glycolipid nmoles/mg cell protein mean ± SE (n)					Ganglioside μg sialic acid/mg cell protein mean ± SE (n)
	GL_1	GL_2	GL_3	GL_4	Total	Total
Fibroblasts						
FD	4.18 ± 0.79 (15)	2.31 ± 0.53 (15)	10.34 ± 1.70[b] (18)	3.53 ± 0.69 (15)	20.36	3.66 ± 0.48[c] (26)
Control	2.34 ± 0.55 (11)	2.45 ± 0.45 (12)	3.20 ± 0.51 (11)	2.16 ± 0.31 (10)	12.67	5.59 ± 0.97 (14)
Fabry	0.70	4.97	18.00	2.56	26.23	
Lymphoblasts						
FD	0.87 ± 0.25 (9)	1.26 ± 0.38 (9)	1.71 ± 0.33[b] (9)	0.19 ± 0.04 (9)	4.03	0.90 ± 0.35[d] (7)
Control	1.01 ± 0.47 (6)	1.77 ± 0.41 (6)	0.54 ± 0.11 (6)	0.33 ± 0.05 (6)	3.65	1.52 ± 0.26 (15)

[a] Familial Dysautonomia (FD) cell lines were grown, and GSL and gangliosides extracted and quantitated as described in the Materials and Methods.

[b] Elevated over control; $0.005 < P < 0.01$.

[c] Decreased below control; $0.025 < P < 0.05$.

[d] Decreased below control; $0.05 < P < 0.10$.

of histological distortion (Figs. 1C and 1D) had higher levels of GL_3 than the line which was close to normal (Fig. 1B). GL_3 levels ranged from 0.83 to 3.18 and 0.17 to 0.83 nmoles/mg cell protein for FD and normal lymphoblasts, respectively. Data on Fabry cell GSL are included for comparison (22).

Since GSL were quantitated by HPLC, the increase in the GL_3 peak may have represented an increase in a GSL other than GL_3, sharing the same retention time, such as GA_2 (asialoGM_2). GM_2 was hydrolyzed to yield GA_2 (33), which was examined on TLC alone and mixed with GSL extracted from cells. GA_2 can be separated from GL_3 on TLC and was not observed in the FD or control cells (Fig. 2). The peak emerging in HPLC as benzoylated "GL_3" from the FD cells, benzoylated GL_3 from control cells, and benzoylated standard GL_3 were collected, debenzoylated (27), digested with α-galactosidase (28) and examined by TLC. The R_f values of the three bands were identical. Benzoyl groups remain on the -NH- of the ceramide portion of the dewbenzoylated GL_3, making it a poor substrate for α-galactosidase, causing it to migrate slightly more slowly than underivatized GL_3. All three lipids were converted to GL_2 to the same limited extent, suggesting that they contained a terminal α-galactosyl linkage (Fig. 3). One hundred percent of underivatized GL_3 was converted to GL_2 (results not shown).

Gangliosides were quantitated by both colorimetric and fluorometric assays (see Materials and Methods). Fibroblast studies were carried out on cells from one to four T75 flasks in an attempt to obtain consistent results. The fluorescent method for measuring gangliosides was found tedious, time-consuming (2 d) and not significantly more sensitive as compared to the resorcinol method which took much less time to complete (one hour). Contrary to the literature, the fluorescent method did not become linear until a ganglioside lower limit of 0.5 μg/mL, close to the resorcinol sensitivity. There was an average 40% significant decrease in the ganglioside content of FD cells, from 5.59 to 3.66, and from 1.52 to 0.90 nmoles/mg in fibroblasts and lymphoblasts, respectively (Table 1). The amount of ganglioside was also quantitated by measuring the specific incorporation of NaB^3H_4 into cellular ganglioside fractions following periodate treatment (see Materials and Methods). This incorporation was 50% diminished, at $0.025 < P < 0.05$ and $0.01 < P < 0.025$ for fibroblasts and lymphoblasts, respectively.

The major ganglioside component of fibroblasts was GD_3, followed by GM_3 and a ganglioside which was neither GM_2 nor GM_1, but with a mobility intermediate between the two. Fibroblasts do not have the UDP-galNAc transferase enzyme necessary to synthesize GM_2 from GM_3 (34). The ganglioside in this area might have been NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcCer (MG-3, or MG-4) which occurs in serum (35). We have designated this ganglioside as '$GM_{2\text{-}1}$,' until exact identification can be made.

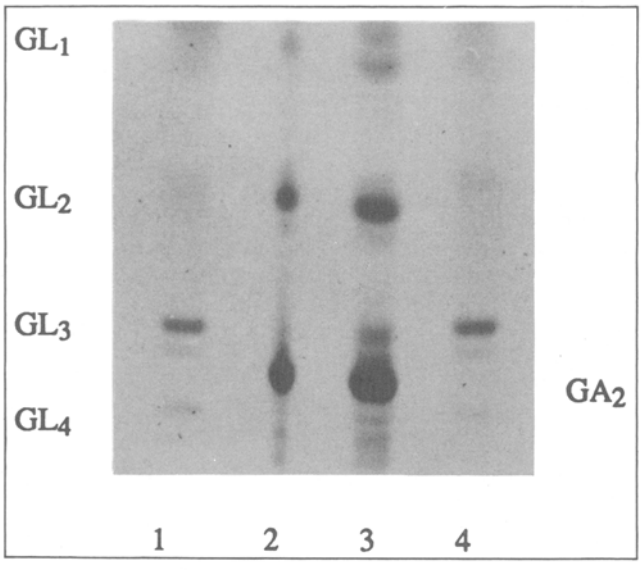

FIG. 2. Investigation of fibroblasts for the presence or absence of asialoGM_1GalNAc(β1-4)Gal(β1-4)GlcCer (GA_2). Fibroblast neutral glycosphingolipids (GSL) fractions and GA_2 were analyzed by thin-layer chromatography (TLC) as described in the Materials and Methods. Lanes 1, 2, 3, and 4 represent neutral GSL extracted from Familial Dysautonomia (FD) cells, a GA_2 preparation, FD cell GSL extracts with added GA_2, and GSL extracted from control cells, respectively. The GSL spotted per lane were not normalized to represent an equivalent amount of total cell protein. The positions of the standards are indicated at the side.

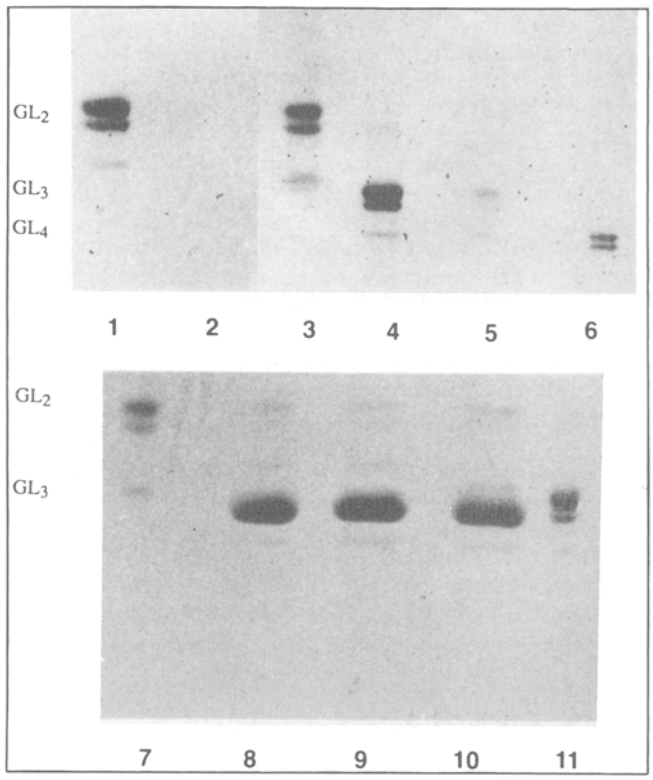

FIG. 3. High-performance thin-layer chromatography (HPTLC) analysis of benzoylated and debenzoylated globotriaosylceramide, Gal(α1-4)Gal(β1-4)GlcCer ("GL_3") from Familial Dysautonomia (FD) and control cells. Lanes 1 to 11 represent, in order, standard lactosylceramide, Gal(β1-4)GlcCer (GL_2), benzoylated FD "GL_3" at the starting line, standard GL_2, standard GL_3, debenzoylated FD "GL_3," standard globotetraosylceramide, GalNac(β1-4)Gal(α1-4)-gal(β1-4)GlcCer (GL_4), standard GL_2, debenzoylated FD cell "GL_3" after reaction with α-galactosidase, debenzoylated control cell GL_3 after reactin with α-galactosidase, debenzoylated standard GL_3 after reaction with α-galactosidase, standard GL_3.

The major ganglioside of lymphoblasts was GM_3. Cells possessed minute quantities of ganglioside in the 'GM_{2-1}' and the GD_3 regions. FD cell ganglioside composition did not differ from that of normal cells.

The ganglioside compositions of conditioned media from FD and control fibroblasts were also similar. Examination of fresh medium containing 10% FCS (as used for growing cells) revealed a complement of gangliosides much the same as conditioned media from FD and control cells, and somewhat similar to human plasma, the major component being GM_3, followed by GD_3, MG-3 and -4 (35) (see above), GM_2, and occasionally GD_{1a}, GD_{1b}, and GT_{1b} (35,36). Thus an HPTLC resorcinol study of gangliosides is non-informative in media containing FCS.

The conent of GSL in conditioned FD or normal media (HPLC), expressed per mL medium (0.28, 0.12, 0.37, and 0.07 nmoles for GL_1 to GL_4, respectively) or per mg cell protein (5.62, 1.43, 14.47, and 2.70 nmoles for GL_1 to GL_4, respectively), did not reveal any major changes. However, published data on the neutral GSL content of FCS revealed that the amount of GSL in medium containing 10% FCS was within range of the levels we found (0.08, 0.05, 0.04, and 0.01 nmoles/mL for GL_1 to GL_4, respectively) (37). Thus neutral GSL, as well as gangliosides, could not be determined meaningfully in the presence of FCS. Lipoprotein-deficient serum (38) still contained GSL (HPTLC results not shown). Accordingly, confluent cells were incubated in roller bottles in serum-free α-MEM medium containing 1% HL-1 Supplement (see Materials and Methods). Only secreted glycolipids, previously masked by the serum GSL, were present.

The total neutral GSL in all conditioned media from FD and control cells remained similar (0.03, 0.02, 0.01 and 0.01 nmoles/mL or 0.48, 0.46, 0.66 and 0.20 nmoles/mg cell protein for GSL GL_1 to GL_4, respectively). When ganglioside fractions were studied on HPTLC, often no resorcinol positive bands were visible, nor was it possible to quantitate the gangliosides using resorcinol *in vitro* as the HL-1-containing medium always interfered. Therefore, the extracts were labelled with NaB^3H_4 which can specifically label only gangliosides (see Materials and Methods), and the incorporation of radioactivity into the samples was measured. Ganglioside fractions from FD cells incorporated three to five times the radioactivity of those fractions from normal cells, suggesting an increase in gangliosides released into the medium in FD.

Since an elevation in GL_3 implies aberrant enzyme activity(s), some lysosomal enzymes were assayed. Our data indicated normal activities for the following lysosomal hydrolases: β-glucosidase, β-hexosaminidase, β-galactocerebrosidase, α-neuraminidase, α-galactosidase (using artificial 4-methylumbelliferyl substrates), and both GM_1 β-galactosidase and β-galactosidase (using natural substrates). Peroxisomal function, assayed as lignoceroyl acid oxidation, appeared intact.

The α-galactosidase enzyme activity, assayed with the natural or artificial substrate, would appear normal even when activator deficienct (29), as the detergent used performs the function of an activator. Therefore FD, Fabry, and control and cells were preloaded with radioactive GL_3, 0.5 nmoles and 340,000 cpm per mL, for one day in serum-free medium to avoid dilution of the GL_3 with unknown amounts of cold GL_3 present in the serum. The extent to which radioactive GL_3 was taken up by the various cell types did not differ, as noted by Kobayashi *et al.* (26) in their study of Fabry's disease. After one day, the radioactive medium was replaced with regular medium, and the percent of the loaded GL_3 hydrolyzed by the cells was measured (see Materials and Methods) on a *per diem* basis, over a period of one to four days. There was a tendency for the percent hydrolysis (7.8, 1.0 and 8.8 for FD, Fabry and control, respectively) and the rate of appearance of radioactive galactose in the medium (72, 9 and 90 pmoles/mg/day for FD, Fabry and control cells, respectively) to be lower in FD cells (averaging data from all the lines listed in the Materials and Methods) than in an average of four control cell lines. These studies were complicated by the fact that FCS itself has α-galactosidase activity, which would degrade any GL_3 released intact from the cells. In parallel studies mirroring those of Kobayashi *et al.* (26), cells were incubated with radioactive GL_3 in medium containing 10% FCS for a period of up to 6 d, and the same parameters determined. Since the serum activity itself accounted for from 25 to 50% of the liberated radioactive galactose, the experiments could not be interpreted when looking for small changes.

These results allude to an alteration in the synthesis and/or degradation of certain GSL and/or gangliosides in FD. We are currently investigating biosynthetic and degradative pathways for neutral GSL and gangliosides in FD, including hydrolases and transferases to see how they relate to this problem. Since GSL contribute to the organization and function of the plasma membrane, a change in GSL composition might affect the organization of other integral and associated glycoproteins, some of which are involved in promoting cell-cell and cell-substratum interactions and adhesions. Our studies were done on fibroblasts and lymphoblasts as nervous tissue is not available for study. The pertinence of similar neuronal membrane component changes to nervous tissue differentiation, reception, synaptogenesis and function is inestimable, and may be involved in the etiology of FD.

In Fabry's disease, denoted by α-galactosidase deficiency and storage of GL_3, there have been many reported indications of impaired autonomic function, such as impaired pupillary response to pilocarpine, altered gastrointestinal function, dimished tear and saliva formation, loss of the cutaneous flare response to scratch and histamine, absence of thermal fingertip corrugations, and preferential loss of myelinated and unmyelinated nerve fibers. This dysfunction is generally attributed to storage of GL_3 in dorsal root and peripheral autonomic ganglia (39–41). The impairments resemble those found in FD (1–3). An overabundance of GL_3 in plasma membranes may be equivalent to 'storage' in other organellar locations and contribute to these impairments in autonomic function. Whereas Fabry's disease is an X-linked disorder distinct from FD, the associated autonomic dysfunction and the biochemical correlation is intriguing.

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Lipid Composition of Subcellular Membranes from Larvae and Prepupae of *Drosophila melanogaster*

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Subcellular membranes were analyzed for their lipid composition and protein content at two developmental points representing the third instar wandering larvae and prepupal stages of *Drosophila*. At both stages, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were the major constituents with phosphatidylinositol (PI), phosphatidylserine (PS), diphosphatidylglycerol (DPG) and phosphatidic acid (PA) being relatively minor components. In total homogenates and in the nuclear-enriched fraction there was no significant difference in the phospholipid composition of the wandering larvae and prepupae. In mitochondria only a significant increase in the minor component PS was observed in the prepupae. In lysosomal membranes on the other hand, the relative abundance of the major components PE and PC increased in the prepupae although the molar ratios of the two lipids remained almost constant. The fatty acid composition of the phospholipids remained virtually unchanged in all of the fractions examined, including the lysosomes, and there was no evidence of lipid peroxidation. With regard to cellular degeneration and the involvement of lysosomes, we conclude that mechanisms other than gross modification of the lipid and/or lipid/protein ratio of their membranes are involved in the liberation of the acid phosphatase contents.
Lipids 27, 984-987 (1992).

In recent years extensive lipid analyses have been reported on a wide variety of insect classes (1). The majority of these reports have concentrated on the analysis of whole organisms and a few on more specific organs, such as the fat body and haemolymph (2). To date, only a limited amount of work on the lipid composition of subcellular fractions has been undertaken. The *Diptera* to which *Drosophila* belongs are known to contain relatively high levels of phosphatidylethanolamine (PE) (3); this to some extent is unusual in as much as phosphatidylcholine (PC) is generally regarded to be the major phospholipid component in most insects (2) and other animals (4), as well as in plants (5). In the current study we analyzed the subcellular lipid composition of *Drosophila melanogaster* at two stages in the life cycle, namely the third instar wandering larvae and the prepupae. Between these two developmental stages, extensive breakdown of the larval tissues is initiated, and the role of lysosomes and their acid phosphatase compliment has been well documented in the cellular disintegration process (6,7). Lipid peroxidation of the lysosomal membranes due to the action of free radicals on the polyunsaturated acyl constituents may damage the membrane and make it more leaky to the acid phosphatase (8,9). Here we report on the lipid composition of several membranes, including the lysosomes, and draw conclusions on the possible role of lipid peroxidation in the mechanism of lysosomal rupture.

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Abbreviations: DAG, diacylglycerol; DPG, diphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; GLC, gas-liquid chromatography; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; RSA, relative specific activity; TAG, triacylglycerol; TLC, thin-layer chromatography; UFA, unesterified fatty acids.

MATERIALS AND METHODS

Insects. Stock wild type *Drosophila melanogaster* were reared in sterilized glass bottles containing a standard ready-mix *Drosophila* medium (purchased from Philip Harris Biological, Weston-super-Mare, U.K.). A yeast pellet was placed on the food surface to aid fermentation. The cultures were maintained at 23 ± 1°C with a light-dark cycle of 12 h. Physical criteria were used to stage the organism (10). Some 8 h before pupariation begins the third instar larva stops feeding and crawls out of the medium, this is known as the wandering larval stage. Pupariation begins with the formation of the white prepupa with its everted spiracles and the cessation of movement. These stages are very distinct and can be readily categorized.

Subcellular fractionation. The isolation of individual organs for subcellular analyses is impractical because of size and, in addition in the prepupal stage, there is extensive tissue degeneration. For these reasons, whole wandering larvae and prepupae (between 70-100 individuals, equivalent to 90-130 mg fresh weight) were used and were hand homogenized using a Potter-Elvehjelm homogenizer in 30 vols (wt/vol) of Tris-HCl (20 mM, pH 7.0), sucrose (0.25 M), ethylenediaminetetraacetic acid (EDTA, 1 mM) and protease inhibitors (1 mg/L leupeptin, 10 mg/L pepstatin and 80 mg/L phenylmethylsulfonylfluoride), on ice. The homogenate was filtered through 4 layers of miracloth (Calbiochem, La Jolla, CA) centrifuged for 10 min at 1,000 × *g*, and the resultant pellet (nuclear enriched fraction) was washed and recentrifuged. The combined supernatants were then centrifuged for 10 min at 10,000 × *g* yielding a mitochondrial fraction which was washed and repelleted as above. An 18,000 × *g* pellet was then obtained from the remaining supernatants and was regarded as the lysosomally-enriched pellet. The post 18,000 × *g* supernatant was also retained for enzyme assays.

Enzyme assays. All assays were performed at 25°C and were completed within 3-4 h following subcellular fractionation. Succinate dehydrogenase was assayed using *p*-iodonitrotetrazolium violet (11) and acid phosphatase using *p*-nitrophenyl phosphate (12) with the addition of L-homoarginine (1 mM) to the incubation medium to inhibit alkaline phosphatase. The glucose 6-phosphatase assays were performed essentially as described (13). The peroxisomal marker enzyme, catalase, is generally unstable and was, therefore, always assayed first by monitoring hydrogen peroxide degradation at 240 nm (14).

Lipid extraction. Lipid samples of homogenates and subcellular fractions in buffer (0.1 M phosphate, pH 7.2; 1 mL) were extracted by adding 0.15 M acetic acid (1 mL)

and chloroform/methanol (1:2, vol/vol; 7.5 mL) followed by gentle mixing. After 2 min, chloroform (2.25 mL) and water (2.25 mL) were added and rapid phase separation was achieved by low-speed centrifugation yielding a lower chloroform layer containing the complex and neutral lipids (modified from Bligh and Dyer, ref. 15). The chloroform phase was reduced to dryness under nitrogen and the residue dissolved in a small volume of chloroform. The polar and neutral lipids were purified by thin-layer chromatography (TLC) on pre-coated silica gel plates (Silica Gel 60; Merck, Darmstadt, Germany) with chloroform/methanol/acetic acid/water (170:30:20:7, by vol) and hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol), respectively. In the polar solvent system, the phosphatidic acid and the diphosphatidylglycerol (DPG) co-migrate. Lipids were located on the TLC plates by lightly staining with iodine vapor and then removed from the plates for transmethylation and fatty acid analysis. Fatty acid methyl esters were prepared in dry methanol containing 2.5% sulfuric acid and were quantified by gas-liquid chromatography (GLC) on an EGGS-X (15% on 100/120 mesh, Chromosorb W-AW, Supelco Inc., Bellefonte, PA) column at 180°C using heptadecanoic acid as the internal standard. Co-chromatography with authentic methyl ester standards (Sigma Chemical Co., Poole, Dorset, U.K.) was used to identify the sample components. Protein determinations were made using the dye binding method outlined in the Biorad assay kit (Bulletin 1069; Biorad, Hemel Hempstead, U.K.).

RESULTS

Subcellular fractionation. Homogenates of wandering larvae and prepupae were subcellularly fractionated by differential centrifugation to yield nuclear (1,000 × *g*), mitochondrial (10,000 × *g*) and lysosomal (18,000 × *g*) enriched fractions. The post 18,000 × *g* supernatant was also collected and assayed. The relative specific activities and yields of a number of marker enzymes were assessed in the different fractions; the results are presented in Table 1.

The bulk of the cellular protein at both stages of development resided in the post 18,000 × *g* supernatant (71–74%); 13–20% was present in the nuclear fraction, 7–11% in the mitochondria, and 4–5% in the lysosomes. The relative specific activity (RSA) of acid phosphatase, as expected, was highest in the lysosomal fractions while that of succinate dehydrogenase was highest in the mitochondria. Contamination of the lysosomes with mitochondria was low although significant levels of acid phosphatase were detected in the mitochondrial fraction. This probably reflects lysosomal heterogeneity (16) in addition to the presence of free acid phosphatase (see ref. 7). The highest yield of both catalase (peroxisomal marker) and glucose 6-phosphatase (endoplasmic reticulum marker) was in the post 18,000 × *g* supernatant; although variations in the RSA in the different fractions were observed, both enzyme activities were low in the lysosomes. Thus, the lysosomes obtained at both stages of development were relatively enriched in the characteristic marker, acid phosphatase and were generally low in contamination from other membrane fractions.

Phospholipid composition of subcellular membranes. The phospholipid composition of the total homogenate extract and of the various subcellular fractions obtained from it are given in Figure 1 (a–d). The major membrane phospholipid in all fractions and at both stages of development was phosphatidylethanolamine (PE). It generally constituted between 50–60% of the total phospholipids with the exception of the lysosomal membranes where it was somewhat lower with values between 35–50%. In the homogenate (Fig. 1a), the next most abundant phospholipid was PC which represented 20–25% of the total, with phosphatidylinositol (PI), phosphatidylserine (PS) and DPG/phosphatidic acid (PA) making up the rest. In the homogenate and nuclear fractions, no significant differences were found in any of the phospholipid compositions between the wandering larvae and prepupae. Similarly, in mitochondria only a significant increase in the relative proportion of the minor component, PS, was observed in the prepupae. In the lysosomes, a wider range of variability in the phospholipid compositions was observed even within a single stage of development compared with that seen in other fractions. The relative abundance of PE (and to a lesser extent, PC) in the membrane

TABLE 1

The Distribution of Marker Enzymes in Subcellular Fractions from Third Instar Wandering Larvae and Prepupae[a]

Fraction	Protein content (%)	Acid phosphatase		Succinate dehydrogenase		Catalase		Glucose 6-phosphatase	
		RSA	yield (%)	RSA	yield (%)	RSA	yield (%)	RSA	yield (%)
Wandering larvae									
Nuclear	12.5	1.3	15.6	2.2	26.7	0.5	5.6	0.6	7.8
Mitochondria	10.5	2.3	23.9	4.5	47.1	0.6	6.1	0.8	8.0
Lysosomes	4.5	3.9	17.7	0.3	1.6	0.8	3.5	1.2	5.4
Supernatant[b]	73.5	0.5	37.3	0.2	16.2	1.0	73.5	1.0	73.1
% Recovery	101.0		94.5		91.6	88.7		94.3	
Prepupae									
Nuclear	19.5	0.6	12.6	2.4	47.7	0.1	2.6	1.3	24.4
Mitochondria	7.4	1.2	8.9	4.8	35.8	1.5	49.1	1.8	13.3
Lysosomes	4.0	1.8	7.4	0.4	1.5	0.9	3.8	0.2	3.8
Supernatant[b]	70.5	0.9	61.6	0.1	8.6	2.2	34.4	0.2	67.5
% Recovery	101.4		90.5		93.6		89.9		109.0

[a]Values are averages of three determinations. RSA, relative specific activity.

[b]Supernatant obtained post 18,000 × *g*.

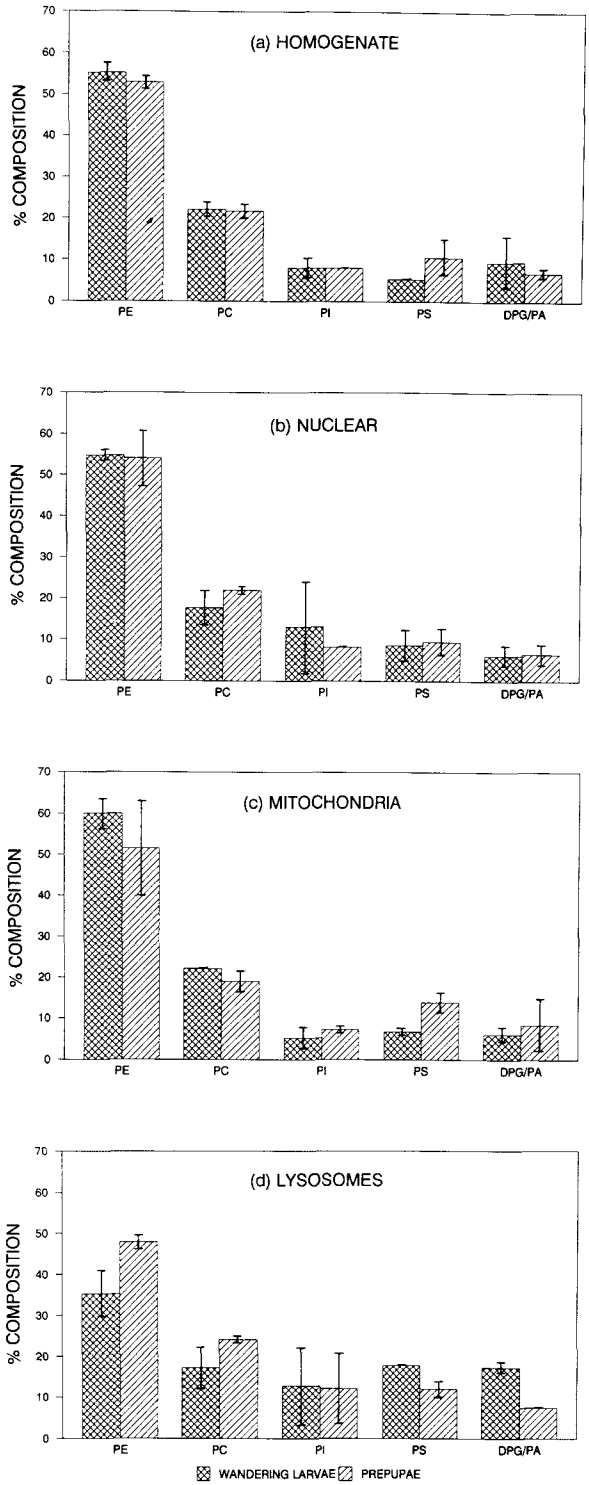

FIG. 1. Phospholipid composition of homogenates and subcellular fractions from wandering larvae and prepupae. Cross-hatched bars, wandering larvae; diagonal bars, prepupae; SEM (n = 3).

increased during the transition to the prepupal stage while that of PS and DPG/PA decreased. On the other hand, the molar ratio of the two major phospholipids, PE and PC, was similar during both stages of development in the lysosomes (2.0 ± 0.1) as well as in the homogenate (2.5 ± 0.1) and mitochondria (2.7 ± 0.1). In the nuclear fraction, however, the ratio was higher in wandering larvae (3.1 ± 0.2) than in prepupae (2.5 ± 0.1). Similarly the protein/phospholipid ratio of the membranes changed little during development. In the mitochondria and lysosomes, the ratios were 2.0 ± 0.1 and 2.4 ± 0.1, respectively. The highest ratios were observed in the nuclear fraction, being in the range of 5.6 ± 0.2.

Fatty acid composition of phospholipids and neutral lipids. The fatty acid composition of the phospholipids in the homogenate and in the lysosomes derived from it was almost identical; for this reason the data for the lysosomes alone are presented in Table 2. Generally, at both stages of development the major acyl constituents were palmitate (16:0), oleate (18:1) and linoleate (18:2) although palmitoleate (16:1) was particularly abundant in PC. PE contained higher levels of 18:1 than 18:2 whereas the reverse was true for PC, PI and DPG/PA. Fatty acids of chain lengths shorter than sixteen carbons, as well as stearate (18:0), gamma-linolenate (γ18:3) and alpha-linolenate (α-18:3) were relatively minor components in most phospholipids except PI where substantial levels of α18:3 were observed. Again, acyl constituents of chainlengths greater than eighteen carbons were generally low in abundance with the exception of DPG/PA lipids where these constituted major components. Based on GLC retention times, the major component of this fraction was tentatively identified as a twenty-carbon unsaturated fatty acid which eluted ahead of arachidonate (20:4). No significant differences between the acyl composition of the phospholipids at the two stages could be found (n = 6 determinations).

The major component of the lysosomal neutral lipids was storage triacylglycerols (TAG, 90%) with diacylglycerol (DAG) and unesterified fatty acids (UFA) and sterols making up the bulk of the remainder. The relative abundance of each of these neutral lipid classes was almost constant at both stages, indicating little degradation of TAG. In both stages of development, typical values for the ratio of phospholipid/neutral lipid were, for homogenate 0.12 ± 0.02, for the nuclear fraction 0.26 ± 0.02, for mitochondria 0.82 ± 0.04, and for lysosomes 0.35 ± 0.05. The acyl composition of the neutral fraction was markedly different from that of the phospholipids and was particularly rich in 14:0 (22.4 ± 1.4%), 16:0 (20.9 ± 0.5%), 16:1 (23.1 ± 1.1%) and 18:1 (19.0 ± 0.2%) with minor proportions of 18:2 (6.8 ± 0.5%), 18:0 (0.7 ± 0.2%), α18:3 (1.3 ± 0.2%) and acyl groups of chainlength greater than eighteen carbon (4.9 ± 1.6%; n = 8, ±SD). Again, there was no significant difference between the acyl composition of the neutral fraction at either stage of development.

DISCUSSION

The major membrane phospholipid in all subcellular fractions of larvae and prepupae of *Drosophila melanogaster* is PE, and high levels of this constituent are generally characteristic of the *Diptera* as a whole (3). From the wandering larvae to the formation of the prepupa, there was little change in the phospholipid composition of the subcellular fractions. The reported ratios, by weight, of phospholipids to protein were in the range commonly encountered for lysosomal membranes (17) and changed little during the transition from the third instar larvae to prepupae. The presence of extensive amounts of neutral

TABLE 2

Fatty Acid Composition of Phospholipids from Lysosomes of Wandering Larvae and Prepupae[a]

Phospholipid	Fatty acid (mol%)								
	<16	16:0	16:1	18:0	18:1	18:2	γ18:3	α18:3	>18[b]
Wandering larvae									
PE	1.1	20.3	8.6	2.6	35.9	19.5	N.D.	2.8	8.7
PC	3.2	15.6	16.9	1.3	21.0	34.1	0.5	3.0	3.6
PI	2.6	16.5	2.5	4.6	20.9	39.4	0.1	9.6	3.0
PS	0.5	7.1	3.1	6.9	44.3	31.3	1.5	1.6	3.2
DPG/PA	0.5	6.7	6.2	3.2	14.1	36.5	N.D.	2.3	29.6
Prepupae									
PE	1.2	23.1	7.3	2.7	34.1	18.7	N.D.	4.2	7.3
PC	2.6	16.0	14.8	1.6	22.2	32.1	0.4	3.9	5.2
PI	0.9	19.9	2.2	2.7	22.0	39.0	N.D.	11.2	0.9
PS	2.2	11.3	3.2	6.8	43.2	26.3	0.2	3.0	2.3
DPG/PA	2.8	7.9	5.8	3.4	15.1	29.3	N.D.	3.9	29.9

[a]Values are average of six analyses. N.D. = not detected. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; DPG/PA, diphosphatidylglycerol/phosphatidic acid.

[b]Tentatively identified as a C_{20} unsaturated fatty acid.

lipids in all fractions (predominantly TAG) almost certainly results from the disruption of the fat bodies in the larvae and the subsequent non-specific binding of these lipids to the subcellular membranes.

In the lysosomes, the relative increase in PE (which in pure form exists in hexagonal phase type II) is offset by the relative increase in PC (a bilayer forming lipid). In this regard, it is the ratio of such lipids which is important in maintaining membrane stability in terms of bilayer structure (18). If lipid peroxidation had played a major role in disrupting lysosomal membrane integrity, then one would have predicted significant decreases in the relative levels of the eighteen carbon polyunsaturated acyl constituents of the membranes in prepupae. The observation that the levels of 18:3 and 18:2 (the constituents most prone to peroxidation) remained almost unchanged in the major lipid components argues against this possibility. Recent evidence (19) indicates that phospholipase A_2 preferentially hydrolyzes peroxidized fatty acid esters in phospholipid membranes as part of a general repair mechanism. Whether such mechanisms operate in *Drosophila* tissue was not investigated in the current study. However, the relatively low levels of PA and the virtual absence of lysophospholipids indicated that little or no phospholipase D and A activities damaged the lysosomes during cell fractionation as has been reported for other preparations (17).

The constancy, therefore, of the membrane parameters investigated at these two developmental points leads us to conclude that mechanisms other than lipid peroxidation or changes in lipid composition are responsible for the disruption of these organellar membranes which are associated with cell degradation.

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Free Fatty Acids Inducing Mouse Lethal Toxicity in Lipid Extracts of *Engraulis japonica*, the Japanese Anchovy

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Mouse lethal toxicity was detected in the ether extract of *Engraulis japonica* (anchovy). The mouse toxicity of extracts was more potent from viscera than from other organs. Okadaic acid ($C_{44}H_{68}O_{13}$) and dinophysistoxin ($C_{45}H_{70}O_{13}$), lipophilic toxins derived from phytoplankton, which are usually considered to be the diarrhetic shellfish toxins, were not detected in the ether extract of ancovy. There occurred, however, two prominent peaks in high-performance liquid chromatography, which were identified as free eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The mouse toxicity observed correlated with the intensity of these two peaks. Toxicity was reduced considerably by pretreatment with Na_2CO_3. By quantitating EPA toxicity, it was concluded that the toxicity was not due to EPA only but also to DHA. The results indicate that substances in Japanese anchovy associated with mouse lethal toxicity include free polyunsaturated fatty acids, mainly EPA and DHA.
***Lipids* 27, 988-992 (1992).**

Bivalves purchased locally in Japanese fishing villages sometimes contain a toxin acquired from marine plankton (1,2), which causes diarrhea in humans. The main active components of this lipophilic diarrhetic toxin have been shown to be okadaic acid (OA) (3) and dinophysistoxins (DTX) (4-6). Since these toxins also have mouse-lethal activity, the presence of the toxin in bivalves has traditionally been monitored by the mouse toxicity test.

While studying the toxicity of bivalves in this laboratory, the presence of a mouse-lethal toxin which was different from the diarrhetic toxins was noted (7). Takagi *et al.* (8,9) reported that major lipophilic toxic substances in the digestive gland of poisonous scallop were polyunsaturated acids. In testing the hemolytic activity to mice of the ether extract of toxic bivalves in our laboratory, the extract was found to change oxyhemoglobin to methemoglobin. Authentic OA at one mouse toxicity unit was, however, found to lack such activity toward oxyhemoglobin. Through further study (10), a group of polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), became suspect as major active agents of the mouse lethal toxins which differ from OA and DTX.

We now report a study on these novel diarrhetic toxins associated with the lipophilic fraction of fish and on their chemical structure.

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Abbreviations: AA, arachidonic acid; ADAM, 9-anthryl diazomethane; DHA, docosahexaenoic acid; DTX, dinophysistoxins; EPA, eicosapentaenoic acid; HPLC, high-performance liquid chromatography; LA, linoleic acid; LNA, linolenic acid; MU, mouse unit; OA, okadaic acid ($C_{44}H_{68}O_{13}$); PBS, phosphate buffered saline; PUFA, polyunsaturated fatty acids; SDA, stearidonic acid; TLC, thin-layer chromatography; UV, ultraviolet.

MATERIALS AND METHODS

Fresh and dried fish were collected from local markets. Fish used in the experiment were *Trachurus japonicus* (Japanese mackerel), *Sebastolobus macrochir* (Kichiji rockfish), *Loligo loligo bleckeri* (squid), *Katsuwonus pelamis* (Arctic bonito), *Cololabis saira* (mackerel pike) and *Engraulis japonica* (Japanese anchovy). In the present study, all tissues in the abdominal cavity, including the mesentery, were used as viscera. Male mice (Funabashi Farm Co. Ltd., Chiba, Japan) weighing 18-20 g were used for the toxicity tests.

Superspecial grades of methanol, *n*-hexane and acetonitrile (Wako Pure Chemical Ind. Ltd., Tokyo, Japan) were used for high-performance liquid chromatography (HPLC). Other chemicals used were of special grades. ADAM (9-anthryl diazomethane) was purchased from the Funakoshi Pharmacy Co. Ltd. (Tokyo, Japan). Linoleic acid (18:2, LA), linolenic acid (18:3, LNA), stearidonic acid (18:4, SDA), arachidonic acid (20:4, AA), EPA (20:5) and DHA (22:6) used as reference materials were from Sigma Chemical Co. (St. Louis, MO) and were of 99% or better purity. Authentic OA and dinophysistoxin-1 (35-methylokadaic acid, DTX_1) purified from cultured cells of the dinoflagellate, *Prorocentrum lima*, and mussels, *Mytilus edulis*, respectively, were provided by Professor Yasumoto of Tohoku University (Sendai, Japan).

DL-α-tocopherol (Wako) was used as antioxidant in an experiment aimed at avoiding PUFA autoxidation. EPA of 92% purity containing 0.2% DL-α-tocopherol, provided by Dr. Hamazaki of Toyama Medical and Pharmaceutical University, Toyama, Japan, was also used in a mouse toxicity tests.

Silica gel plates ($60F_{254}$, 20 cm × 20 cm and 20 cm × 5 cm, 0.5 mm thickness, E. Merck, Darmstadt, Germany) were heated for 2 h at 110°C for activation and kept in a desiccator until use.

The HPLC instrument and the fluorescence detector used for the assay of OA and DTX_1 were a Jasco FLC-A20 (Nihonbunko Ind. Ltd., Tokyo, Japan) and a Jasco 820FP, respectively. Recorders used were the Pantos U-228 (Nippondensi Co. Ltd., Tokyo, Japan) and the Shimadzu C-R3A (Shimadzu Co. Ltd., Kyoto, Japan).

For treatment with ADAM before HPLC, ordinary Seppak silica cartridges (Waters Associates, Milford, MA) were utilized. The HPLC and the ultraviolet (UV) spectrophotometric detector for the determination of EPA and DHA in anchovy were the Shimadzu LC-6A and SPD-6A, respectively; a Shimadzu C-R6A was used as recorder.

Extraction of lipophilic toxin from various fish. Lipophilic toxin was extracted according to the method previously described for the preparation of shellfish lipophilic toxin (4). One-hundred grams of various fish (80 g for viscera) were homogenized in 300 mL of acetone. After filtration, the extraction was repeated twice with 400 mL acetone. After three extractions, the acetone was combined and evaporated, and the residue was further extracted with 200 mL of diethyl ether.

Crude lipid. A crucible, in which the ether extract corresponding to one gram wet weight of fish was placed, was dried at 120°C for 1 h and was left to cool to room temperature in a desiccator for 30 min. The difference in weight was considered the weight of crude lipid.

Mouse bioassay. The test solution for the mouse bioassay was prepared from the crude lipid so as to represent 4 g of fish in 1 mL phosphate buffered saline (PBS, pH 7.4) containing 1% Tween 60.

Based on the definition (11) that one mouse unit (MU) was the amount of toxin injected intraperitoneally (i.p.) to kill a mouse weighing 20 g within 24 h, the toxicities of test solutions from fish were measured. In the present paper, samples showing less toxicity than 0.25 MU/g were coded as not detectable (ND).

Determination of OA and DTX_1 in various fish. The assay to determine OA and DTX_1 was performed according to the method of Lee *et al.* (12). Ether extracts from 5 g of fish were dissolved in 5 mL of methanol and mixed vigorously; 100 μL of the methanol solution was evaporated under N_2, and 0.1 mL of 0.1% ADAM in methanol was added. After esterification by standing in the dark at room temperature for 1 h, the ADAM reaction mixture was placed on a Sep-pak cartridge. The column was washed with 5 mL of *n*-hexane/chloroform (1:1, vol/vol) and eluted with 5 mL of pure chloroform. Finally, a fraction of ADAM-esterified OA and DTX_1 was obtained with 5 mL of chloroform/methanol (95:5, vol/vol). After removing the solvent under N_2, the residue was dissolved in 0.1 mL of methanol, of which 20 μL was submitted to HPLC. The HPLC assay conditions were as follows: Column, ODS (4.6 mm i.d. × 250 mm, Jasco Fine-pak Sil C_{18}, Nihonbunko); mobile phase, acetonitrile/methanol/water (8:1:1, vol/vol/vol); flow rate 1.1 mL/min; assay wavelengths, Ex = 365 nm, Em = 412 nm. Peak areas were calculated by integration with the C-R3A recorder. HPLC peaks were identified by comparison of retention times with those of authentic standards in two different solvent systems, namely acetonitrile/methanol/water (8:1:1, vol/vol/vol), and methanol/water (95:5, vol/vol).

Fractionation of lipids from the ether extract of anchovy viscera by thin-layer chromatography (TLC). Ether extracts containing 0.16 g of lipids from anchovy viscera (0.5 MU/g toxicity), with α-tocopherol added (0.5%) to minimize oxidation throughout the extraction procedure, were applied onto four silica gel plates (20 cm × 20 cm), and the plates were developed with petroleum ether/diethyl ether/acetic acid (74:15:1, vol/vol/vol) as described by Ozawa *et al.* (13). Lipids were thus fractionated into five zones as shown in Figure 1. Fraction (Fr.) 1 consisted mainly of phospholipids (at origin); Fr.2 contained two bands (R_f 0.06 and 0.13); Fr.3 contained mainly free fatty acids (R_f 0.26); Fr.4 contained mainly triglycerides (R_f 0.43); and Fr.5 consisted of two bands of esters (R_f 0.73 and 0.80) including cholesteryl esters. The bands were extracted as follows. The fractions between phospholipids and free fatty acids were extracted three times with 30 mL of methanol. Other lipids such as triglyceride and cholesteryl ester were extracted three times with 30 mL of diethyl ether. After evaporating the solvents under N_2, test samples for the mouse bioassay were prepared by adding 3.0 mL of PBS to each lipid fraction dissolved in 0.2 mL of methanol.

Concentrations of free EPA and DHA in anchovy viscera. The total concentrations of free EPA and DHA were determined by HPLC following a modification of the method by Ozawa *et al.* (14). Ether extracts of anchovy viscera were prepared at a concentration of 9.4 mg wet weight of viscera/mL in methanol, and 10 μL of the sample was submitted to HPLC. The concentrations of EPA and DHA in the samples were determined from calibration curves. The HPLC assay conditions were as follows: Column, ODS (4.6 mm i.d. × 250 mm, Shim-pack CLC ODS(M), Shimadzu); mobile phase, acetonitrile/methanol/water/phosphoric acid (53:20:17:0.1, by vol); flow rate, 1.2 mL/min; assay wavelength, 195 nm. The identity of the EPA and DHA peaks in HPLC was confirmed by capillary gas chromatography.

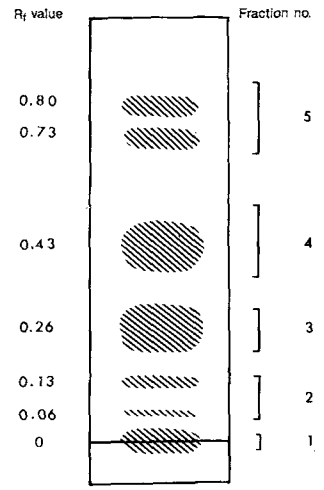

FIG. 1. Thin-layer chromatogram of the ether extracted lipids (0.5% α-tocopherol added) from anchovy viscera corresponding to 0.5 MU/g toxicity. Solvent: petroleum ether/diethyl ether/acetic acid (74:15:1, vol/vol/vol).

Mouse lethal toxicity of EPA of 92% purity. To examine whether the EPA concentrations found in anchovy would be lethal to mice, standard EPA (92% pure) containing 0.2% α-tocopherol was tested for mouse toxicity. A stock solution of 0.5 g of EPA dissolved in 2 mL of methanol was prepared, and was serially diluted with PBS containing 1% Tween 60 (pH 7.4) to give concentrations of 25, 12.5, 6.3 and 3.2 mg per 0.2 mL. Each sample (0.2 mL) was injected i.p. into each of four mice. The solvent, 0.2 mL of 50% methanol, showed no mouse toxicity upon i.p. injection.

RESULTS

The mouse toxicity of the crude lipids extracted from the fish examined is shown in Table 1. Phospholipids were present at relatively low levels because acetone was used for lipid extraction. Extracts of the fresh fish, *i.e. Trachurus japonicus, Sebastolobus macrochir* and *Loligo loligo bleckeri,* had lipid contents in the range of 1.4–5.6%, and pH values between 3.5–4.1. Extracts from muscles and viscera of *Katsuwonus pelamis* gave 1.1 and 1.5% lipid, and pH values of 3.5 and 3.6, respectively. Fresh viscera of *Cololabis saira* contained 33.3% lipid. Fresh muscle and viscera of *Engraulis japonica* had lipid contents in the range 1.1–5.6% and pH values between 3.1 and 4.5. The lipid percentages and pH values were 3.3 and 3.0 for dry whole anchovy, and 7.5 and 3.1 for dry viscera,

TABLE 1

Characteristics of Ether Extracts from Various Fish

Fish	Lipid (%)	pH	Mouse toxicity (MU/g)[a]
Trachurus japonicus (fresh, whole)	5.6	3.5	ND[b]
Sebastolobus macrochir (fresh, whole)	3.6	3.7	ND
Loligo loligo bleckeri (fresh, whole)	1.4	4.1	ND
Katsuwonus pelamis (fresh, muscle)	1.1	3.5	ND
(fresh, viscera)	1.5	3.6	ND
Cololabis saira (fresh, viscera)	33.3	—	ND
Engraulis japonica (fresh, whole)	1.1	3.6	ND
(fresh, whole)	5.6	3.1	ND
(fresh, whole)	3.6	4.5	ND
(fresh, whole)	1.2	4.0	0.25
(fresh, viscera)	1.6	3.7	0.25
(fresh, viscera)	2.1	3.7	0.25
(fresh, viscera)	2.6	3.7	0.50
(fresh, viscera)	0.8	3.5	0.50
(dry, whole)	3.3	3.0	0.25
(dry, viscera)	7.5	3.1	1.00

[a]Mouse lethal units/g wet weight. [b]Not detectable.

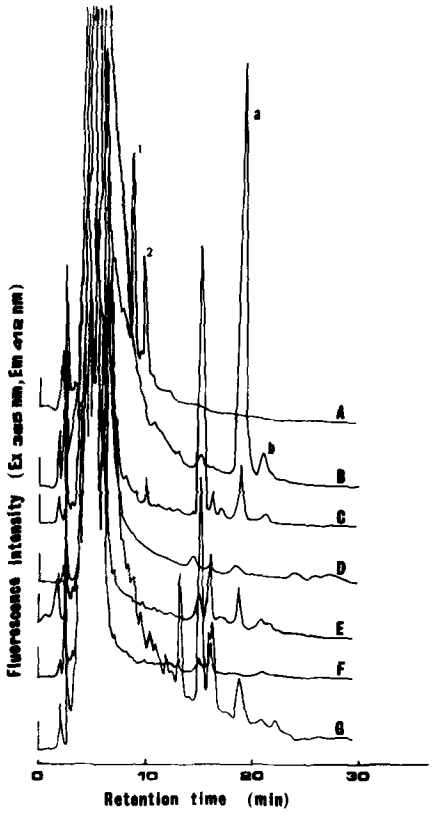

FIG. 2. High-performance liquid chromatography (HPLC) tracing of 9-anthrylmethyl esters of okadaic acid, dinophysistoxin-1, and of extracts from various fish. Fractions eluted with chloroform/methanol (95:5 vol/vol) corresponded to 20 mg fish homogenate when submitted HPLC. A, 15.3 ng okadaic acid (peak 1) and 13.3 ng dinophysistoxin-1 (peak 2); B, *Engraulis japonica*, viscera; C, *Sebastolobus macrochir*, whole; D, *Cololabis saira*, viscera; E, *Trachurus japonicus*, whole; F, *Loligo loligo bleckeri*, whole; G, *Katsuwonus pelamis*, viscera. Peaks a and b were identified as eicosapentaenoic acid and docosahexaenoic acid, respectively, based on HPLC analysis and comparison with authentic standards. Assay conditions: Column, ODS (4.6 mm i.d. × 25 cm); solvent, acetonitrile/methanol/water (8:1:1, vol/vol/vol); flow rate, 1.1 mL/min; wavelength, Ex = 365 nm, Em = 412 nm.

respectively (Table 1). Mouse toxicity was observed only in anchovy, *Engraulis japonica*, especially in the viscera and in the dried product. No mouse toxicity was detected in the other fish. Mice injected with 1 mL of test solution extracted from anchovy with 1 MU/g toxicity died within 1–3 h; those injected with ether extract possessing a toxicity of less than 0.5 MU/g died between 10 and 24 h after injection. Symptoms were immobilization and crouching down, but not diarrhea.

To see whether oxidation of lipid (*i.e.*, PUFA) throughout the pretreatment phase would affect mouse toxicity, α-tocopherol (0.5%) was added to a parallel sample of fresh anchovy, showing 0.5 MU/g, before extraction with acetone. No change was apparent in mouse lethal toxicity by the addition of α-tocopherol.

Crude lipid contents were high in *Trachurus japonicus*, *Sebastolobus macrochir* and *Cololabis saira*, which showed no mouse toxicity. In anchovy, samples with high lipid content did not always show high mouse toxicity, and samples containing less lipid often showed a higher toxicity. Almost all ether extracts from the fish showed acid pH (3.0–4.5).

Since OA and DTX_1, which have been identified as acidic toxins in bivalves, presumably originate from phytoplankton, detection of these acidic agents was attempted from anchovy, which is basically a plankton feeder (15), with zooplankton typically making up 70% and phytoplankton making up 30% of the diet. OA and DTX_1 were not detected by HPLC in any of the fish examined (Fig. 2).

An unknown substance (marked a in Fig. 2) was eluted at a retention time of 18.6 min from ether extracts of anchovy viscera. A small amount of this substance was also observed in other fish but not in the squid *Loligo loligo bleckeri*. Also detected was another unknown substance at a retention time of 14.9 min which occurred at the same level in *Katsuwonus pelamis* viscera and in *Sebastolobus macrochir*.

The concentrations of the substance a in viscera from three samples of Japanese anchovy were 4.8, 5.7 and 10.1 times higher than those in the respective whole bodies. The amounts of substance a as represented by the peak areas and the mouse toxicities of the respective six samples of anchovy viscera are listed in Table 2. The mouse toxicities appear to correlate quite well with the amounts of substance a present. Changes upon treatment with Na_2CO_3 to remove acidic substances are shown in Table 3. Remarkable decreases in the amounts of substance a detected by HPLC and in mouse toxicities were observed while total lipids decreased much less (Table 3).

Because substance a reacted with ADAM, and based on its polarity in HPLC, the substance was considered likely to be a free fatty acid. By comparison with authentic free fatty acid standards, using two different HPLC solvent systems, the substances with retention times of 18.6 min (a) and 21 min (b) (Fig. 2) were identified as EPA

TABLE 2

Mouse Toxicity and Peak Areas of Substance a in an Ether Extract from Anchovy Viscera

Sample	Mouse toxicity (MU/g)[a]	Integrated peak area of substance a at HPLC RT 18.6 min (× 10^4)[b]
1	0.25	41.6
2	0.25	67.9
3	0.25	127.6
4	0.5	156.7
5	0.5	214.5
6	1.0	282.5

[a]Mouse lethal units/g wet weight.

[b]HPLC, high-performance liquid chromatography; RT, retention time.

TABLE 3

Effect of Na_2CO_3 Treatment of an Ether Extract from Anchovy Viscera

Na_2CO_3 treatment (0.5 M)	Lipid (%)	pH	Toxicity (MU/g)[a]	Integrated peak area of substance a at HPLC RT 18.6 min (× 10^4)[b]
−	7.5	3.1	1.0	282.5
+	6.6	7.4	ND[c]	24.7

[a]Mouse lethal units/g wet weight.

[b]HPLC, high-performance liquid chromatography; RT, retention time.

[c]Not detectable.

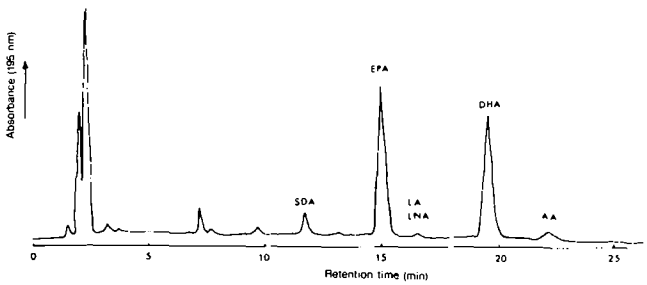

FIG. 3. High-performance liquid chromatography (HPLC) tracing of methanol-soluble substances in an ether extract from anchovy viscera corresponding to 1.0 MU/g of mouse toxicity. A fraction corresponding to 94 μg anchovy viscera was submitted to HPLC. LA, linoleic acid; LNA, linolenic acid; SDA, stearidonic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Assay conditions: Column, ODS (0.46 × 25 cm); solvent, acetonitrile/methanol/water/phosphoric acid (53:20:17:0.1, by vol); flow rate, 1.2 mL/min; wavelength, 195 nm.

and DHA, respectively. A spectrum of the ether extract of anchovy viscera analyzed by HPLC with a UV detector is shown in Figure 3. It is apparent that EPA and DHA in their free fatty acid form were the main components detected in the anchovy viscera lipid extract.

To examine the accuracy of the present method for the detection of EPA, 1 mg of EPA was added to 1 mL of the ether extract from anchovy viscera containing 0.25% EPA, and the mixture was assayed. The recoveries for

TABLE 4

Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) Concentrations in Ether Extracts from Anchovy Viscera with Various Toxicities

Sample	EPA (mg/g)[a]	DHA (mg/g)[a]	Mouse toxicity (MU/g)[a]
1	7.25	5.7	1.0
2	3.39	3.8	0.25
3	3.36	2.5	0.25
4	1.27	1.7	ND[b]

[a]As wet weight of anchovy viscera. [b]Not detectable.

EPA in duplicate experiments were 101 and 103%. Reproducibility for the same sample in six experiments was within 2.4%. To confirm with which lipid class mouse lethal toxicity was associated, 1 mL of each of the samples of the five lipid classes isolated by TLC (Fig. 1) was injected i.p. into two mice. The mice injected with the free fatty acids (Fr.3) were dead within 24 h; all others survived.

The free EPA and DHA concentrations in the ether extracts of four anchovy viscera, whose mouse lethal toxicities were 1.0, 0.25, 0.25 and ND, are shown in Table 4. DHA levels were only slightly lower than those of EPA. The mouse lethal toxicities, however, correlated better with the EPA concentrations. This was tested with authentic EPA (92% pure). Upon injection with 25 mg, 12.5 mg, 6.3 mg and 3.2 mg of authentic EPA into four mice each, 4, 4, 1 and 0 mice, respectively, died within 24 h. Thus 12.5 mg of EPA was considered to correspond to approximately 1 MU.

DISCUSSION

It had previously been reported that lipid fractions from bivalves can be toxic to mice and can change oxyhemoglobin to methemoglobin when added to blood *in vitro* (7). In the present study, the presence of a similarly toxic substance was examined in several species of fish. Remarkably, the toxicity was detected only in ether extracts from anchovy viscera, although this material contained lower lipid concentrations than did other fish, such as *Cololabis saira, Trachurus japonicus* and *Sebastolobus macrochir.* The result suggested that the substance associated with mouse toxicity was specific for the anchovy.

Because mouse toxicity was reduced by treatment with Na_2CO_3, the toxic substance was presumed to be acidic. Because mouse toxicity correlated with the amount of ADAM-reacted EPA measured (Table 2), and EPA was notably decreased by treatment with Na_2CO_3 (Table 3), EPA appeared to be the cause of mouse lethal toxicity. As shown in Table 3, the reduction of EPA after Na_2CO_3 treatment as determined by HPLC was 91.3%, while that of total lipid was only 12.0%. This is consistent with the observation that free fatty acid levels in sardines are quite low (1.9–3.2%) (16).

The fact that more ADAM-reacted EPA was detected in anchovy than in other fish, in spite of the ancovy having a low lipid content, suggested the presence of more EPA in free acid form. Takagi *et al.* (8,9) had previously reported that free unsaturated fatty acids, including EPA,

were more toxic than the corresponding methyl esters. The amount of EPA corresponding to 1 MU, calculated from toxicity tests on extracts from anchovy viscera, was 7.25 mg (Table 4) when EPA alone was considered toxic. Based on use of authentic EPA, 1 MU was about 12.5 mg. These data are close to those reported by Takagi *et al.* (8), who correlated 1 MU with 6–12 mg. These authors also reported (8,9) that LNA and AA had mouse lethal toxicity equal to EPA, and LA and DHA were nearly half as toxic as EPA.

Enzymic oxidation of fish lipids *in vivo* is known to promote the formation of undesirable volatile carbonyl compounds (17). Cyclooxygenase (18) and lipoxygenase (19,20) were recently discovered in the gill and skin of teleost fish, such as trout and turbot. In the barnacle, *Elminius modestus,* 8-hydroxyeicosapentaenoic acid (8-HEPE), an oxidative metabolite of EPA, was identified as a highly active hatching factor (21). However, toxicity was absent in our Fr.2 separated by TLC (Fig. 1), which would contain the fatty acid oxidation products. Although enzymic oxidative metabolites, including eicosanoids, should not be ignored as substances inducing mouse lethal toxicity, PUFA appear to be the major cause of mouse lethal toxicity in the present study.

The reason why toxicity was higher in dry fish than in the fresh fish can probably be attributed to the fact that PUFA were liberated enzymatically from the more complex lipids of viscera *post mortem.* Free EPA and DHA in anchovy actually increased with the time elapsed after death (data not presented).

Mouse assays have been widely used in place of chemical assays for detecting the agents that cause human diarrhea, *i.e.* OA and DTX from bivalves. From the present study we can conclude that the presence of free EPA, DHA and similar PUFA can appreciably confound the mouse assay.

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Palmitic Acid Enhances Cholesterol Gallstone Incidence in Sasco Hamsters Fed Cholesterol Enriched Diets

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In an established hamster model of cholesterol cholelithiasis, a semipurified lithogenic diet containing 4% butterfat and 0.3% cholesterol leads to the production of cholesterol gallstones in only 50–60% of animals after a 6-wk feeding period. The purpose of this study was to investigate whether gallstone incidence could be increased while feeding a nutritionally adequate diet of moderate cholesterol content. The semipurified lithogenic diet was modified as follows: (i) substitution of 1.2% palmitic acid for 4% butterfat, and (ii) varying the amount of dietary cholesterol from 0.0 to 0.3% with either butterfat or palmitic acid as the lipid component of the diet. Substitution of palmitic acid for butterfat produced a significantly higher incidence of cholesterol gallstones (94% *vs.* 53%). Palmitic acid also raised the incidence of gallstones when added to the 0.1% and 0.2% cholesterol diets as compared to butterfat: 0% *vs.* 44% and 50% *vs.* 81%, respectively. Gallstone incidence increased from 0% to nearly 100% when the cholesterol content of the palmitic acid diets was raised from 0.0% to 0.3%, indicating a dose response effect with respect to dietary cholesterol. Hamsters fed cholesterol-free diets did not form gallstones. Increased dietary cholesterol led to increased liver weight associated with a significant increase in liver cholesterol concentration. However, the palmitic acid groups had significantly lower liver cholesterol values than the corresponding butterfat groups. Serum and biliary cholesterol concentrations increased with increasing dietary cholesterol intake, but there were no differences between the butterfat and palmitic acid groups. The cholesterol saturation index increased from 0.56 to 1.32 in the butterfat groups and from 0.56 to 1.30 in the palmitic acid groups upon raising the dietary cholesterol from 0.0 to 0.3%. Biliary total bile acid concentration did not vary significantly within all groups; however, the addition of cholesterol produced an increase in the ratio of chenodeoxycholic acid to cholic acid. It is concluded that in Sasco hamsters the saturated fatty acid, palmitic acid, when substituted for butterfat in a nutritionally adequate lithogenic diet, is capable of increasing gallstone incidence to almost 100% during a 6-wk feeding period.
Lipids 27, 993–998 (1992).

Many animal models of cholesterol cholelithiasis have been described in the literature (1–17). The hamster has been used extensively because its bile composition has many similarities to that of humans. Although several hamster models have been described, none was entirely satisfactory. For example, Dam and Christensen (13–15) used a fat-free, nutritionally inadequate diet that inhibited normal growth and resulted in premature deaths. Some "strains" of hamster tend to form pigment rather than cholesterol gallstones, and in other "strains" the incidence of gallstones can be very low (12,17–19). The diet of Pearlman *et al.* (16) required dietary cholesterol and ethinyl estradiol, and, for unexplained reasons, the model lost its reproducibility as noted in several laboratories.

Recently, we developed a hamster model of cholesterol cholelithiasis using a nutritionally adequate diet of moderate cholesterol content (12). We employed a semipurified diet (SPD) containing 0.3% cholesterol leading to the production of pure cholesterol gallstones with an incidence of 50–60%. The male golden Syrian hamster from Sasco Inc. (Omaha, NE) was chosen since this "strain" gave a satisfactory and a reproducible incidence of cholesterol gallstones (12). The hamsters exhibited normal weight gain and were healthy throughout the 6-wk experimental period. However, the proportion of animals developing cholesterol gallstones never approached 100% with this diet. In the present manuscript, we investigated ways of raising gallstone incidence while feeding a nutritionally adequate diet.

The semipurified diet (SPD) contained 4% butterfat, which has a very high proportion of saturated fatty acids varying in chain length from C_4 to C_{18}. We previously showed that butterfat was an important dietary ingredient for gallstone formation in our model (20). Palmitic and oleic acids are major fatty acids in butterfat (21). Substitution of olive oil (71% oleic acid) for butterfat prevented stone formation completely even in the presence of 0.3% dietary cholesterol (22). However, substitution of palm oil (45% palmitic acid) for butterfat enhanced cholesterol gallstones (84%, Cohen, B.I., and Mosbach, E.H., unpublished data). Therefore, we hypothesized that removal of the monounsaturated acid would further enhance gall-stone formation. We therefore modified the SPD by substituting palmitic acid for butterfat and studied the effect of this diet on gallstone formation. In addition, we also carried out a dose response experiment to examine the effect of varying the dietary cholesterol from 0.0 to 0.3% with either butterfat or palmitic acid as the lipid component of the diet.

The data suggest that palmitic acid plays an important role in cholesterol gallstone formation in the male golden Syrian hamster from Sasco, since substitution of palmitic acid for butterfat produced a higher incidence of cholesterol gallstones (94% *vs.* 53%). There was a dose response effect with respect to dietary cholesterol: gallstone incidence increased from 0 to nearly 100% when the cholesterol content of the palmitic acid diet was raised from 0.0 to 0.3%.

MATERIALS AND METHODS

Animals. Male golden hamsters *(Mesocricetus auratus)*, weighing 53–68 g, were purchased from Sasco Inc. The animals were maintained with Purina rodent chow and water *ad libitum* for a 1-wk quarantine period prior to the start of the experimental diets. The animals were divided

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Abbreviations: alloCA, allocholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FT-IR, Fourier Transform Infrared; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; MDCA, murideoxycholic acid; PAD, palmitic acid diet; SPD, semipurified diet.

into 8 groups and fed the following diets (purchased from Dyets Inc., Bethlehem, PA): group 1, SPD containing 43.7% corn starch, 20% casein, 14.6% dyetrose (soluble starch), 10% fiber (cellulose), 5% salt mix (modified U.S.P. XIV salt mix, no. 200951), 4% butterfat, 2% corn oil, 0.5% vitamin mix (no. 300000), 0.2% choline chloride, 0% cholesterol; group 2, SPD with 0.1% cholesterol; group 3, SPD with 0.2% cholesterol; group 4, SPD with 0.3% cholesterol; group 5, palmitic acid diet (PAD), 0% cholesterol, equivalent to SPD with 1.2% palmitic acid substituted for 4% butterfat; group 6, PAD with 0.1% cholesterol; group 7, PAD with 0.2% cholesterol; group 8, PAD with 0.3% cholesterol. The hamsters were fed the experimental diets for 6 wk. The food intake was monitored; water was supplied *ad libitum*. At the end of the 6-wk feeding period, the hamsters were fasted for 24 h and anesthetized with 20 mg of ketamine hydrochloride (Aveco Co., Inc., Fort Dodge, IA). Blood was withdrawn by cardiac puncture for the determination of serum cholesterol. The gallbladder was examined for gallstones. Bile was removed with a 50 μL Hamilton Syringe and examined under a polarizing light microscope (Olympus MCHAP microscope, Olympus Corp., Lake Success, NY) for the presence of liquid crystals or cholesterol crystals. The remaining bile was immediately aliquoted for the determination of biliary lipids. The liver was excised, weighed, and a portion was taken for cholesterol analysis.

Analytical procedures. Biliary bile acids were determined as the methyl ester acetates using an SPB-5 15 m capillary column (Supelco, Bellefonte, PA) attached to a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA); 3α,7α-dihydroxy-12-keto-5β-cholanoic acid was used as an internal standard (9). Biliary phospholipids were determined by an enzymatic-colorimetric procedure using the Wako phospholipid B kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cholesterol concentrations of bile, serum and liver were analyzed by gas-liquid chromatography of the trimethylsilyl ether derivative, using 5α-cholestane as an internal standard (23). Gallstones were analyzed by Fourier Transform Infrared (FT-IR) spectroscopy on a Perkin-Elmer model 1710 spectrometer (Norwalk, CT) attached to a model 7500 laboratory computer for data storage and analysis.

Calculations and statistics. The cholesterol saturation index of bile was determined using published methods (24,25). The data were reported as the mean ± SD. Differences of gallstone incidence between the various groups were determined using chi-square analysis (26,27). Other differences among groups were calculated using one-way analysis of variance followed by Student's *t*-test (26,27).

RESULTS

All hamsters were fed the experimental diets for 6 wk. In all 8 groups, there were no significant differences in food intakes, which ranged from 9 to 12 g/d. The animals remained healthy and gained similar weights throughout the experiment; cholesterol feeding led to increased liver weights (Table 1).

The incidence of gallstones and biliary cholesterol crystals was determined at sacrifice (Table 2). The hamsters fed the cholesterol-free SPD (group 1) formed neither gallstones nor cholesterol crystals. The addition of 0.1% cholesterol to the SPD (group 2) also failed to elicit the production of gallstones. Formation of cholesterol gallstones (50%) and crystals (50%) occurred with the addition of 0.2% cholesterol to the SPD (group 3). The highest and usual dose of cholesterol (0.3%) added to the SPD (group 4) did not significantly increase the incidence of gallstones (53%) and crystals (65%) from that in group 3. Substitution of palmitic acid for 4% butterfat in the SPD without cholesterol (PAD, group 5) failed to induce gallstones. However, the addition of 0.1% cholesterol to PAD (group 6) increased the formation of gallstones (44%) and crystals (62%) from the corresponding SPD with butterfat (group 2). Increasing dietary cholesterol to 0.2% (group 7) caused the formation of gallstones (81%) and crystals (94%) to increase still further. The highest dose of cholesterol (0.3%) added to the PAD (group 8) further increased gallstones (94%) and crystals (100%). FT-IR analysis found the gallstones to consist mainly of cholesterol (<90%); palmitic acid was not a constituent of the gallstones. In all cases, the palmitic acid diets plus cholesterol resulted in significantly more stones and crystals than the corresponding SPD butterfat diets (groups 6–8 *vs.* groups 2–4, respectively).

TABLE 1

Hamster Data: Body Weight, Food Intake and Liver Weight[a]

Group	Diet	Number of animals	Initial weight (g)	Final weight (g)	Food intake (g/d)	Liver weight (g)
1	Semipurified diet (SPD)	14	57 ± 3	94 ± 7	10 ± 1	3.8 ± 0.4[b]
2	SPD + 0.1% cholesterol	16	60 ± 3	101 ± 10	10 ± 1	5.5 ± 0.8
3	SPD + 0.2% cholesterol	16	60 ± 2	109 ± 7	11 ± 0	6.5 ± 1.0
4	SPD + 0.3% cholesterol	17	60 ± 4	102 ± 10	11 ± 1	6.2 ± 0.6
5	Palmitic acid diet (PAD)	10	53 ± 4	88 ± 16	9 ± 1	3.9 ± 0.5[c]
6	PAD + 0.1% cholesterol	16	59 ± 3	95 ± 15	10 ± 1	5.3 ± 1.1
7	PAD + 0.2% cholesterol	16	59 ± 3	100 ± 8	11 ± 0	5.5 ± 0.5
8	PAD + 0.3% cholesterol	16	61 ± 4	100 ± 7	12 ± 1	6.0 ± 0.6

[a]Animals were fed the experimental diets as indicated. See Materials and Methods for details of the semipurified and palmitic acid diets. Numbers are mean ± SD.
[b]Differs significantly from groups 2–4, $P < 0.01$.
[c]Differs significantly from groups 6–8, $P < 0.01$.

TABLE 2

Cholesterol Gallstones and Crystals in Hamsters at Sacrifice[a]

Group	Diet	Cholesterol gallstones		Cholesterol crystals	
		Number	Percent	Number	Percent
1	Semipurified diet (SPD)	0/14	0	0/14	0
2	SPD + 0.1% cholesterol	0/16	0	0/16	0
3	SPD + 0.2% cholesterol	8/16[b]	50	8/16	50
4	SPD + 0.3% cholesterol	9/17[c]	53	11/17	65
5	Palmitic acid diet (PAD)	0/10[d]	0	0/10	0
6	PAD + 0.1% cholesterol	7/16[e]	44	10/16	62
7	PAD + 0.2% cholesterol	13/16	81	15/16	94
8	PAD + 0.3% cholesterol	15/16	94	16/16	100

[a]See Materials and Methods for details of the semipurified and palmitic acid diets.
[b]Differs significantly from groups 1 and 2, $P < 0.05$.
[c]Differs significantly from group 8, $P < 0.01$.
[d]Differs significantly from group 6, $P < 0.025$.
[e]Differs significantly from group 2, $P < 0.005$.

TABLE 3

Cholesterol Levels in Hamsters at Time of Death[a]

Group	Diet	Liver (mg/g)	Serum (mg/dL)	Bile (mg/mL)
1	Semipurified diet (SPD)	8.22 ± 2.17[b]	197 ± 25[c]	2.30 ± 0.94[d]
2	SPD + 0.1% cholesterol	44.39 ± 7.14[e]	279 ± 37[e]	2.95 ± 1.44
3	SPD + 0.2% cholesterol	66.29 ± 8.52[f]	333 ± 35	3.70 ± 2.26[d]
4	SPD + 0.3% cholesterol	96.57 ± 12.98[g]	344 ± 38[g]	5.46 ± 2.06
5	Palmitic acid diet (PAD)	2.82 ± 0.60[h]	137 ± 14[h]	1.86 ± 0.48[i]
6	PAD + 0.1% cholesterol	21.40 ± 3.59[j]	281 ± 49[j]	4.09 ± 2.33
7	PAD + 0.2% cholesterol	28.93 ± 2.72[g]	343 ± 55[g]	4.99 ± 1.87
8	PAD + 0.3% cholesterol	40.13 ± 8.59	399 ± 55	5.58 ± 2.36

[a]See Materials and Methods for details of the semipurified and palmitic acid diets. Numbers are mean ± SD.
[b]Differs from groups 2-4, $P < 0.01$.
[c]Differs from groups 2 and 5, $P < 0.01$.
[d]Differs from group 4, $P < 0.01$.
[e]Differs from groups 3 and 6, $P < 0.01$.
[f]Differs from groups 4 and 7, $P < 0.01$.
[g]Differs from group 8, $P < 0.01$.
[h]Differs from group 6, $P < 0.01$.
[i]Differs from groups 6-8, $P < 0.01$.
[j]Differs from group 7, $P < 0.01$.

Table 3 summarizes the cholesterol levels in liver, serum and bile. There was a significant increase in liver cholesterol concentration as the dietary cholesterol in the SPD and PAD groups was raised from 0.0 to 0.3%. The PAD groups (5-8) had much lower liver cholesterol concentrations, which ranged from 2.82 to 40.13 mg/g, compared to the corresponding SPD groups (1-4), which ranged from 8.22 to 96.57 mg/g. The serum cholesterol level increased from 197 to 333 mg/dL on the SPD with 0.0 to 0.2% cholesterol (groups 1-3) but did not increase further with 0.3% cholesterol (group 4) (344 mg/dL). In the PAD groups (5-8), the serum cholesterol increased from 137 to 399 mg/dL as dietary cholesterol was raised from 0.0 to 0.3%. Biliary cholesterol also increased as the cholesterol concentration in the diet was raised from 0.0 to 0.3%; however, there were no significant differences between the SPD (2.30-5.46 mg/mL) and the PAD (1.86-5.58 mg/mL) groups.

Biliary lipids and cholesterol saturation indices are summarized in Table 4. As expected, the molar percentage of biliary cholesterol increased as a function of dietary cholesterol content in each group. The mole percent phospholipids did not significantly change as cholesterol was carried from 0.0 to 0.2% in the SPD (groups 1-3) but significantly increased to 20.6% with the 0.3% cholesterol diet (group 4). In the PAD groups, the percent phospholipids increased with 0.2 and 0.3% dietary cholesterol (groups 7 and 8) to 17.7 and 20.9%, respectively, compared to the 0.0 and 0.1% (groups 5 and 6) which both had 14.4%. There were no major differences between the SPD and PAD groups. Molar percent of bile acids decreased when dietary cholesterol increased from 0.0 to 0.3% in the SPD and PAD groups, ranging from 80.8 to 71.2% and 82.8 to 70.5%, respectively. With the addition of cholesterol to the diet, the cholesterol saturation index

TABLE 4

Effect of Diet on Biliary Lipids[a]

Group	Diet	Mole % biliary lipid			Total lipid (g/dL)	Cholesterol saturation index
		Cholesterol	Phospholipid	Bile acid		
1	Semipurified diet (SPD)	3.2 ± 0.5[b]	16.4 ± 1.9	80.4 ± 2.2	9.4 ± 3.0	0.56 ± 0.10[c]
2	SPD + 0.1% cholesterol	5.0 ± 1.5	14.2 ± 3.1	80.8 ± 4.2	7.8 ± 2.9	0.97 ± 0.21[d]
3	SPD + 0.2% cholesterol	6.0 ± 1.4[e]	15.9 ± 2.9	78.1 ± 4.0	7.8 ± 3.9	1.16 ± 0.25
4	SPD + 0.3% cholesterol	8.2 ± 0.9	20.6 ± 3.7[f]	71.2 ± 4.4[f]	9.4 ± 3.2	1.32 ± 0.25
5	Palmitic acid diet (PAD)	2.8 ± 0.3[g]	14.4 ± 2.5[h]	82.8 ± 2.6	9.5 ± 2.4	0.56 ± 0.07[i]
6	PAD + 0.1% cholesterol	5.7 ± 1.3	14.4 ± 3.5	79.9 ± 4.7	9.7 ± 4.6	1.16 ± 0.26
7	PAD + 0.2% cholesterol	6.9 ± 2.0[j]	17.7 ± 2.2[j]	75.4 ± 2.8	10.3 ± 3.5	1.13 ± 0.36
8	PAD + 0.3% cholesterol	8.6 ± 2.4	20.9 ± 3.8	70.5 ± 3.4[k]	9.6 ± 4.1	1.30 ± 0.41

[a]See Materials and Methods for details of the semipurified and palmitic acid diets. Numbers are mean ± SD.
[b]Differs from group 2, $P < 0.01$; group 5, $P < 0.03$.
[c]Differs from groups 2–4, $P < 0.01$.
[d]Differs from group 3, $P < 0.03$.
[e]Differs from group 4, $P < 0.01$.
[f]Differs from groups 1–3, $P < 0.01$.
[g]Differs from group 6, $P < 0.01$.
[h]Differs from groups 7 and 8, $P < 0.01$.
[i]Differs from groups 6–8, $P < 0.01$.
[j]Differs from group 8, $P < 0.03$.
[k]Differs from groups 5–7, $P < 0.01$.

TABLE 5

Effect of Diet on Biliary Bile Acid Composition[a]

Group	Diet	Total bile acid (mg/mL)	LCA (%)	DCA (%)	CDCA (%)	CA (%)	HDCA (%)	Others (%)
1	Semipurified diet (SPD)	56.1 ± 18.0	1.1 ± 0.5	8.8 ± 4.8	23.0 ± 12.6[b]	45.9 ± 5.2[b]	8.0 ± 4.5	13.2 ± 3.1
2	SPD + 0.1% cholesterol	47.5 ± 16.7	2.5 ± 0.8	10.4 ± 3.6	24.4 ± 4.7	42.1 ± 6.7	9.1 ± 4.2	11.5 ± 2.6
3	SPD + 0.2% cholesterol	44.5 ± 20.4	2.0 ± 0.8	7.6 ± 4.1	31.9 ± 7.1[c]	39.3 ± 5.1[d]	9.6 ± 5.6	9.6 ± 2.8
4	SPD + 0.3% cholesterol	49.0 ± 15.4	1.9 ± 1.2	6.9 ± 4.8	42.4 ± 18.2	29.2 ± 6.0	8.9 ± 6.0	10.7 ± 2.3
5	Palmitic acid diet (PAD)	59.7 ± 17.4	1.6 ± 0.2	9.6 ± 2.3	23.3 ± 3.7[e]	43.7 ± 6.1[e]	5.4 ± 0.8	16.4 ± 3.7
6	PAD + 0.1% cholesterol	57.5 ± 25.4	3.0 ± 0.7	11.2 ± 3.3	26.5 ± 2.9	37.7 ± 3.1[f]	11.3 ± 3.1	10.3 ± 3.1
7	PAD + 0.2% cholesterol	62.3 ± 24.2	2.9 ± 0.4	11.1 ± 2.2	27.2 ± 2.7	34.3 ± 3.7	14.9 ± 2.5	9.6 ± 2.6
8	PAD + 0.3% cholesterol	50.4 ± 24.4	2.0 ± 1.2	8.2 ± 5.3	38.2 ± 21.4	33.7 ± 10.5	8.8 ± 6.6	9.1 ± 1.5

[a]See Materials and Methods for details of the semipurified and palmitic acid diets. Numbers are mean ± S.D. LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; HDCA, hyodeoxycholic acid; Others include allocholic acid (alloCA), and murideoxycholic acid (MDCA).
[b]Differs from groups 3 and 4, $P < 0.03$.
[c]Differs from groups 2 and 4, $P < 0.04$.
[d]Differs from group 4, $P < 0.01$.
[e]Differs from groups 6–8, $P < 0.02$.
[f]Differs from group 7, $P < 0.01$.

increased from 0.56 to 1.32 in the SPD groups and from 0.56 to 1.30 in the PAD groups.

Table 5 summarizes the biliary bile acid composition in the gallbladder. The total bile acid concentration did not vary significantly within the 8 groups and averaged 44.5–62.3 mg/mL. The addition of cholesterol produced an increase in the proportion of chenodeoxycholic acid (CDCA) and a corresponding decrease in cholic acid (CA), as was previously observed (12). The gradual increase in CDCA as cholesterol was raised from 0.0 to 0.3% ranged from 23.0 to 42.4% in the SPD and from 23.3 to 38.2% in the PAD.

DISCUSSION

The aim of this study was twofold: (i) to study how the substitution of palmitic acid for butterfat in the diet affects gallstone incidence, and (ii) to examine the dose response of the model to increasing cholesterol intake. Palmitic acid (1.2%) was substituted for 4% butterfat in the SPD to maintain a similar level of palmitic acid in both the SPD and PAD diets. Butterfat contains palmitic acid as a major saturated fatty acid, about 27% of total fatty acids (21).

We determined that both cholesterol and butterfat were essential additions to our SPD hamster diet to achieve the induction of gallstones (20). Butterfat (4%) without cholesterol (group 1) in the diet does not induce gallstones. In addition, no stones were formed with 0.1% cholesterol; however, the incidence was 50% using 0.2% dietary cholesterol. The highest dose of cholesterol (0.3% did not significantly increase stone incidence further. Our hamster model clearly shows a dose response to choelsterol in the induction of the cholelithiasis.

Raising dietary cholesterol from 0.0 to 0.3% increases the proportion of cholesterol relative to bile acids and lecithin in bile which would favor the formation of lithogenic bile (28-31). Since cholesterol is insoluble in bile, an aqueous medium, it is solubilized in mixed micelles composed of bile acids and lecithin (32-34). In addition, excess cholesterol may be carried in the bile in the form of cholesterol-lecithin vesicles. Unlike the micelles, these vesicles may be unstable, and cholesterol is more likely to precipitate when the bile has a high level of such vesicles (35-37).

Gallstone formation increased upon substituting free palmitic acid for butterfat; presumably, the cholesterol solubilizing capacity of bile was reduced. The dietary saturated free fatty acid, palmitic acid, has a definite enhancing effect on gallstone formation. This effect is not ascribed to the presence of free fatty acid *per se* since our unpublished results have shown that the substitution of palm oil (containing 45% palmitic acid) for butterfat (containing 27% palmitic acid) also enhances gallstone incidence. Therefore, the addition of this saturated fatty acid in any form, either free or as triglyceride, seems to enhance gallstone formation. This is also suggested by our results showing that 0.1% cholesterol in the palmitic acid diet produced a gallstone incidence of 44% while the corresponding SPD (butterfat diet) gave a 0% incidence. The 0.2% and 0.3% cholesterol diets containing palmitic acid also produced a higher stone incidence than the corresponding butterfat groups (81% and 94% *vs.* 50% and 53%). These studies suggest that cholesterol gallstone induction results from increased secretion of cholesterol into the bile which can be stimulated in two ways, by increasing the intake of dietary palmitic acid, or reducing the proportion of mono- and diunsaturated fatty acids. It may well be that the oleic acid component of butterfat contributed to the reduced gallstone incidence with butterfat as opposed to palmitic acid. In studies carried out in our laboratory, the substitution of olive oil, which contains 13% palmitic acid and 71% oleic acid, for butterfat, yielded 0% stone incidence with our 0.3% cholesterol diet (22). Apparently, the types of fatty acids in the diet are important in lithogenesis. In our model, saturated fatty acids enhance gallstones incidence, while the unsaturated fatty acids seem to protect against gallstone formation.

As dietary cholesterol was increased from 0.0 to 0.3% in the SPD and PAD, the liver cholesterol levels became significantly elevated. Liver cholesterol ranged from 2.82 to 40.13 mg/g in the PAD groups and from 8.22 to 96.57 mg/g in the SPD groups. The palmitic acid partially reduced the accumulation of cholesterol in the liver. Studies have shown that dietary cholesterol is esterified and stored in the liver cells as relatively inert cholesteryl esters (38). It has further been shown that the cholesteryl esters in the liver increase with increases in the dose of cholesterol (39). Storage of cholesterol in the liver as the ester has been shown to be reduced by certain fats in the diet (40). The ω6 unsaturated fatty acids seem to be the preferred substrates in cholesterol esterification (41). Saturated fatty acids have been shown to be poor substrates for the esterification reaction (42). Since palmitic acid is a saturated fatty acid, this may explain why liver cholesterol levels were lower with PAD than with the butterfat diets, which contain many fatty acids, saturated as well as unsaturated. As a second alternative, the liver cholesterol levels suggest that palmitic acid promotes cholesterol absorption to a lesser extent than butterfat.

In summary, the present data demonstrate that dietary palmitic acid and cholesterol affect gallstone incidence. Specifically, when palmitic acid was substituted for butterfat, there was an increase in cholesterol cholelithiasis. Therefore, a lithogenic diet containing palmitic acid and cholesterol is a potent alternative to feeding a butterfat-cholesterol diet. In the hamster model, the palmitic acid-cholesterol diet is capable of raising gallstone incidence to almost 100% during a 6-wk feeding period. The mechanism of the enhancement of cholesterol cholelithiasis by dietary palmitic acid requires elucidation.

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Fecal Bile Acid Excretion and Composition in Response to Changes in Dietary Wheat Bran, Fat and Calcium in the Rat

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The effect and possible interactive influence of different dietary amounts of wheat bran, fat and calcium on the fecal excretion, concentration and composition of bile acids was studied in Fischer-344 rats. The fecal bile acids were analyzed using gas-liquid chromatography. Dietary wheat bran increased both total bile acid excretion and fecal weight without changes in fecal bile acid concentration. The proportion of fecal hyodeoxycholic acid decreased with increasing dietary fiber, whereas that of lithocholic and deoxycholic acids increased significantly with fiber intake. The percent content of fecal chenodeoxycholic acid did not change. Increasing dietary fat led to an increase in bile acid excretion without changes in either fecal weight or bile acid concentration. In contrast, the level of dietary calcium did not affect the total excretion of bile acids. However, since calcium increased the fecal weight, it consequently diluted bile acids and decreased their fecal concentration. Dietary fat and calcium had no influence on fecal bile acid composition. There were no interactive effects of wheat bran, fat and calcium on fecal bile acids. The finding in this study that dietary fiber, fat and calcium induce significant changes in fecal bile acids may be of relevance to the potential of bile acids to promote carcinogenesis.
Lipids 27, 999-1004 (1992).

Bile acids have been shown to express cancer-promoting properties which are modified by dietary factors (1-18). In particular, fiber, fat and calcium have received increased attention for their ability to induce changes in the metabolism of bile acids which affect their potential for promoting the development of experimental colon cancer (6-18). Whereas both dietary fiber and calcium can suppress the experimental induction of colon cancer by carcinogens (10,12,14-16,18), increased ingestion of fat has the opposite effect (7,13,19).

The potential changes in bile acid metabolism, which are considered to be relevant to the promotion of colon cancer, consist of increases in the fecal excretion and concentration of bile acids as well as their modification by bacteria. Most authors have reported that diets high in any type of fiber lead to both an increase in the total excretion and a suppression in the bacterial degradation of bile acids (6,9,11, 15,20-23), but the effect on fecal bile acid concentration appears to be less certain (11,23-26). Several studies in human subjects indicate that fecal concentrations of bile acids decrease after the ingestion of a diet high in either wheat bran or a mixtue of dietary fibers (24,25). In other human studies that involved diets high in oat bran and citrus pectin, respectively, no decreases in fecal bile acid concentrations were observed (20,22).

On the other hand, a high fat intake has been reported to result in an increased fecal excretion and concentration of bile acids (7,14). Although it is known that calcium binds to bile acids (27,28), little information is available regarding the effect of dietary calcium on fecal bile acids. There also appear to be no systematic studies of the combined or interactive effects of fiber, fat and calcium on bile acids. The aim of the study reported in the present paper, therefore, was to determine the individual effects of different levels of wheat bran, fat and calcium, and the influence of their possible interactions on the fecal excretion, concentration and composition of bile acids.

MATERIALS AND METHODS

Study design and animal groups. Male albino Fisher-344 rats (F344/NHsdBR, Hilltop Lab Animals, Inc., Scottsdale, PA), weighing approximately 190 to 225 g, were used to measure the effects of 36 different experimental diets. The latter were characterized, in a factorial study ($4 \times 3 \times 3$), by the combinations of different concentrations, by weight, of wheat bran (0, 2.5, 10 and 20%), fat (1, 5, 10%), and calcium (0.18, 0.52 and 1.04%). The dietary fat, 1/3 of it saturated, 1/3 monounsaturated and 1/3 polyunsaturated, consisted of 44.2% lard, 46.2% corn oil and 9.6% hydrogenated coconut oil. Calcium carbonate served as the source of calcium in the diets. Two standard additional diets, Purina Rat Chow 5001 and AIN-76A (Purina, St. Louis, MO) served as controls. The calcium content of the Purina Rat Chow is 1%, and that of the AIN-76A diet is 0.52%. The rats were kept for 28 d on these diets. Each of the 38 study groups consisted of 10 animals.

Preparation of diets and collection of fecal samples. The above described combinations of wheat bran, fat and calcium were mixed into a basal diet. There were no other sources of fiber, fat and calcium. The only cholesterol within the diet was derived from the fat mixture. The basal diet had the following composition (g/kg): casein, 198; DL-methionine, 2; choline bitartrate, 2; sucrose, 369 to 269; dextrose, 369 to 269; a vitamin mixture, 10; and a salt mixture, 35. The salt mixture provided the phosphorus to the diets and did not vary. The diets followed the standard guidelines established for experimental animals (29). The diets were produced and pelleted by Dyets Inc. (Bethlehem, PA). The designated composition of the experimental diets was independently verified by analyses which were performed by two other laboratories.

Following a one-week quarantine, the rats were housed in groups of two, under standard conditions of 23°C with a 12-h dark-light cycle, in the core animal facility of The George Washington University Medical Center, Washington, D.C. The animal care was provided according to HHS/NIH guidelines. The respective diets were fed along with water *ad libitum* for 28 d. To ensure an accurate assessment of animal intake, any food that was spilled or uneaten was measured and the weight was subtracted from the total weight of food ration. Three days before the

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Abbreviation: GLM, group least square means.

completion of the diet treatment, the animals were transferred, in groups of two, into metabolic cages which contained a funneled tray for accurate collection of feces, without contamination by urine or food. All feces were collected for 72 h, weighed and then stored in polypropylene test tubes at −80°C overnight. Following lyophilization and mechanical homogenization, the fecal material was reweighed in order to determine the wet/dry ratio. The feces from each paired group were pooled and immediately stored at −20°C until analyzed for fat and bile acid content.

Fecal bile acid analysis. The fecal bile acids were qualitatively and quantitatively analyzed using gas-liquid chromatography, essentially as previously described by this laboratory and others (30-33). In brief, aliquots of fecal material, with nordeoxycholic acid added as an internal standard, were refluxed with absolute ethanol for 30 min, cooled and then decanted through filter paper and collected. This process was repeated using chloroform/methanol (1:1, vol/vol). In order to separate the bile acids from the neutral sterols, the filtrate with the extracted bile acids was evaporated, then redissolved in 15 mL of 0.01 N HCl, and percolated through a prewashed Lipidex column (Lipidex 1000, Packard Instrument Corporation, Downers Grove, IL). The column was then washed with 20 mL of distilled water. The bile acids were eluted with 20 mL of 68% methanol. After elution from the column, the bile acids were subjected to alkaline hydrolysis. The deconjugated bile acids were extracted with dichloromethane, and the bile acid methyl ester acetates were formed as previously described (31-33). The recovery of bile acids in the extraction and deconjugation procedures was evaluated with the use of ^{14}C-labelled bile acids. The recovery was 64 ± 2.66 (M ± SEM) % for lithocholic acid, 79 ± 0.44% for cholic acid and 62 ± 3.77% for chenodeoxycholic acid. The derivatized bile acids were analyzed on a Packard Gas Chromatograph (Packard Instrument Corporation) using a 6-ft coiled column with a cyanosilicone stationary phase (3% AN-600 on Gas Chrom Q; Analabs, Camden, CT).

Fecal fat analysis. The fecal fat content was determined by the method of van de Kamer *et al.* (34).

Fecal calcium analysis. Fecal calcium was assayed by atomic absorption spectroscopy (Varian Techtron AA-5, Palo Alto, CA) using commercially prepared standards (Fisher Scientific, Silver Spring, MD) (35,36). A 0.5% $LaCl_3$ standard was included in all calcium analyses to correct for phosphate interference.

Statistical analysis. The effects of fiber, fat and calcium and their interactions were evaluated by three-way analysis of variance, using the group least square means (GLM) procedure in the SAS statistical computer package (37). GLM were then compared using the conservative Bonferroni adjustment for multiple comparisons (38). Pearson correlations were also computed between selected pairs of variables. The criterion for statistical significance was set at $\alpha = 0.01$.

RESULTS

The overall results of the measurements of body weight and fecal weights and bile acids, as well as fecal fat and calcium content derived from rats in the combined dietary groups are presented in Table 1. The correlations between

TABLE 1

Results of Measurements in Combined Dietary Groups (mean ± SEM)

	Dietary groups[a]											
	Fiber (%)				Fat (%)				Calcium (%)			
	0	2.5	10	20	1	5	10		0.18	0.52	1.04	
Body weight	289.8 ± 1.51	298.0 ± 1.51	305.2 ± 1.81	301.9 ± 1.72	293.5 ± 1.52	301.0 ± 1.45	302.4 ± 1.46[c]		298.8 ± 1.42	298.6 ± 1.57	299.3 ± 1.58	
Fecal measurements												
Wet wt, (g/24 h)	0.49 ± .04	0.66 ± .04	1.05 ± .05	1.73 ± .07[c]	0.96 ± .07	0.96 ± .07	1.03 ± .08		0.65 ± .06	0.99 ± .08	1.32 ± .06[c]	
Dry wt, (g/24 h)	0.49 ± .04	0.59 ± .04	0.91 ± .05	1.40 ± .05[c]	0.87 ± .06	0.86 ± .06	0.91 ± .07		0.64 ± .06	0.85 ± .06	1.12 ± .06	
Total bile acid (μmol/24 h)	5.48 ± .46	8.22 ± .92	15.07 ± 1.21	23.66 ± 2.79[c]	12.37 ± 2.04	14.09 ± 1.47	15.19 ± 1.75[d]		14.53 ± 2.33	13.22 ± 1.57	13.74 ± 1.47	
Total bile acid conc.[b] (μmol/g fecal dry wt)	16.03 ± 2.36	15.11 ± 1.45	16.79 ± 1.15	18.22 ± 2.82	15.30 ± 2.27	17.64 ± 1.47	17.00 ± 1.19		23.70 ± 2.51	15.25 ± 1.08	11.67 ± 1.06[c]	
Total fat (mg/24 h)[b]	24.72 ± 2.66	33.89 ± 2.98	54.99 ± 3.42	91.95 ± 6.04[c]	35.38 ± 2.76	50.23 ± 3.84	69.45 ± 6.16[c]		34.75 ± 3.29	51.06 ± 4.69	69.61 ± 5.26[c]	
Total calcium (mEq/24 h)[b]	14.38 ± 1.77	12.16 ± 1.57	14.57 ± 1.62	16.74 ± 1.67	14.28 ± 1.51	14.29 ± 1.33	14.78 ± 1.48		2.97 ± .19	13.50 ± .54	27.28 ± .91[c]	

[a]Fiber dietary groups are composed of nine subgroups, while fat and calcium dietary groups are composed of twelve subgroups. Each subgroup consists of ten animals, as described in Materials and Methods.
[b]Published as abstract (ref. 50).
[c,d]Symbolizes significant changes with increases from the lowest to the highest level of the indicated diet c, $P < 0.001$; d, $P < 0.05$.

TABLE 2

Correlations Between Fecal Bile Acid Excretion and Fecal Weight, Fat and Calcium in the Different Dietary Groups[a]

	Dietary groups									
	Fiber (%)				Calcium (%)			Fat (%)			All groups combined
	0	2.5	10	20	0.18	0.52	1.04	1	5	10	
Fecal wt, wet (g/24 h)	n.s.	n.s.	0.413[a]	n.s.	0.407[a]	0.635[c]	0.563[c]	n.s.	0.452[b]	0.656[c]	0.454[c]
Fecal wt, dry (g/24 h)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fecal fat (mg fat/24 h)	n.s.	n.s.	0.710[c]	n.s.	n.s.	0.539[c]	0.439[c]	0.400[b]	0.403[b]	0.382[b]	0.356[c]
Fecal calcium (mEq/24 h)	−0.549[b]	n.s.	0.448[b]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

[a]Correlation coefficients which are statistically not significant are not presented (n.s.). [a]$P < 0.01$, [b]$P < 0.005$, [c]$P < 0.001$.

TABLE 3

Correlations Between Fecal Bile Acid Concentration[a] and Fecal Weight, Fat and Calcium in the Different Dietary Groups[a]

	Dietary groups									
	Fiber (%)				Calcium (%)			Fat (%)			All groups combined
	0	2.5	10	20	0.18	0.52	1.04	1	5	10	
Fecal wt, wet (g/24 h)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fecal wt, dry (g/24 h)	−0.70[c]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fecal fat (g/24 h)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fecal calcium (mEq/24 h)	−0.771	n.s.[c]	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	−0.357[b]	n.s.	−0.284[c]

[a]Fecal bile acid concentration per fecal dry weight.
[b]See Table 2, footnote *a*.

the fecal bile acids, and fecal weight, fat, and calcium, respectively, are presented in Table 2 and Table 3. The effect of varying concentration of dietary fiber, fat and calcium upon rat body weight, fecal weight, bile acid concentrations, percent composition of the individual bile acids, as well as total and individual fecal bile acid excretion was analyzed.

Body weight. The average weight gain, which was very similar in the different groups, was approximately 65 g. There was no significant difference in the amount of diet consumed between the different experimental groups. Neither the fiber nor the calcium content of the diet had any appreciable influence on weight gain. However, there was a small, but statistically significant increase in body weight with increasing dietary fat. There was a significant increase ($P < 0.001$) in animal weight in the 10% dietary fat group (Table 1).

Dietary fiber. Fecal wet weight (Fig. 1) as well as fecal dry weight increased significantly with increasing dietary fiber ($P < 0.001$). The total fecal excretion of bile acids increased significantly with increasing dietary fiber (Fig. 2). It increased from a mean value of 5.48 in the 0% fiber group to that of 23.66 mmol/24 h in the animals which received 20% fiber ($P < 0.001$) (Table 1). This significant increase of fecal bile acid excretion with increasing dietary fiber content was seen with lithocholic, chenodeoxycholic, deoxycholic and hyodeoxycholic acids, which constituted the major fecal bile acids (Fig. 3, Table 4). Total bile acid excretion showed significant positive correlations with fecal wet weight ($P < 0.01$), fecal fat content ($P < 0.001$) and fecal calcium excretion ($P < 0.005$), in the 10% fiber group, as well as a negative correlation ($P < 0.005$) with fecal calcium content in the 0% fiber group (Table 2 and Table 3).

The fecal bile acid concentrations, based on both wet and dry fecal weight, were not affected by increasing dietary fiber. With the exception of the 0% fiber group, there was no correlation between fecal bile acid concentration per fecal dry weight or fecal wet weight, and fecal wet weight, dry weight, fat and calcium. In the 0% fiber group, the bile acid concentration showed a significant negative correlation ($P < 0.001$) with fecal dry weight and calcium ($P < 0.001$).

The dietary wheat bran-associated changes in fecal acid composition are shown in Table 4. The proportion of hyodeoxycholic acid decreased significantly ($r = -0.7999$, $P < 0.001$) from a mean value of 50.34% in the 0% fiber group to that of 39.09% on 20% fiber. In contrast, lithocholic and deoxycholic acids increased significantly ($r = 0.7871$, $P < .001$ and $r = 0.7580$, $P < .001$, respectively) with fiber intake. The respective values in the 0% and 20% fiber groups were 1.91 and 15.34% for lithocholic acid and 15.74 and 28.59% for deoxycholic acid. The percent content of fecal chenodeoxycholic acid did not change. Cholic, α-muricholic and β-muricholic acids, which were not present in all animals, did not show significant changes in response to the different diets (Table 4).

Dietary fat. Total bile acid excretion increased signifi-

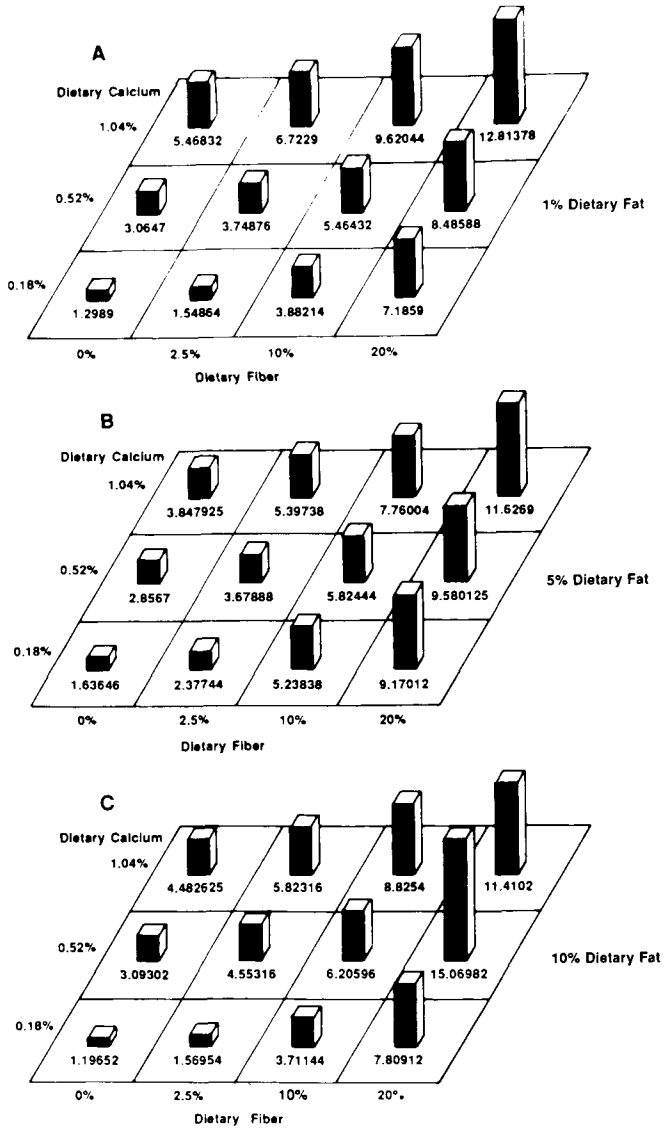

FIG. 1. Effects of dietary fiber, fat and calcium on fecal wet weight (g/24 h). The bars represent the effects of increasing dietary fiber and calcium on fecal wet weight in relation to dietary fat content. Panel A, 1% fat diet; panel B, 5% fat diet; panel C, 10% fat diet.

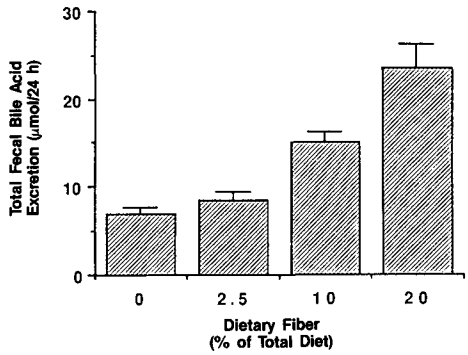

FIG. 2. The effect of increasing dietary fiber content on the total fecal excretion of bile acids.

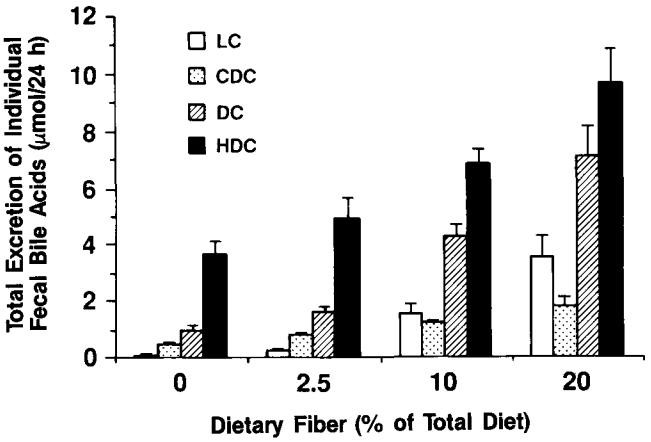

FIG. 3. Effect of dietary fiber on the excretion of the major fecal bile acids. LC, lithocholic; CDC, chenodeoxycholic; DC, deoxycholic; HDC, hyodeoxycholic.

cantly from 12.37 umol/24 h in the 1% fat group to 15.19 μmol/24 h in the animals which received a 10% fat diet ($P < 0.05$) (Table 1). Fecal fat showed significant increases with increasing dietary fat content (Table 1). The fecal wet and dry weights as well as fecal bile acid concentrations and composition were not affected by dietary fat.

Fecal bile acid excretion showed a significant positive correlation with fecal wet weight ($r = 0.452$, $P < .005$ for the 5% fat group and $r = 0.656$, $P < .001$ for the 10% fat group) (Table 2). Significant positive correlations existed also between bile acid excretion and fecal fat in all three dietary fat groups (Table 2). There was a significant negative correlation ($r = -0.357$, $P < .005$) between the fecal bile acid concentration per fecal dry weight and fecal calcium excretion in the 5% dietary fat group (Table 3).

Dietary calcium. Fecal weight (wet) increased significantly ($P < 0.001$) with increasing dietary intake of calcium, *i.e.* from 23.70 μmol/g in the 0.18% calcium group to 11.67 μmol/g in the rats which received a 1.04% calcium diet (Table 1, Fig 4). The total excretion (Table 1) and percent composition of fecal bile acids did not change with dietary calcium.

Correlations in combined dietary groups. Total fecal bile acid excretion (Table 2 and Table 3) correlated significantly with fecal wet weight ($r = 0.4536$, $P < 0.001$) and with fecal fat ($r = 0.3555$, $P < 0.001$).

Total fecal bile acid concentrations showed a significant negative correlation with fecal calcium when the data from all dietary groups were combined ($r = 0.2835$, $P < .001$). Cholic, α-muricholic and β-muricholic acids were not present in all animals and did not show significant changes in response to the different diets (Table 4).

Additive effects of diets. The only additive effect of any combination of the diets was observed with fiber and calcium in relation to increasing fecal wet and dry weight.

DISCUSSION

In agreement with work by others, this study shows that a diet high in wheat bran is associated with significant increases in fecal bile acid excretion (6,9,11,15,20–23). The

TABLE 4

Percent Composition of Fecal Bile Acids in Relation to Fiber Content of Diet (M ± SEM)

Bile acid	Dietary fiber content (%)			
	0	2.5	10	20
Lithocholic	1.91 ± 0.33	4.38 ± 1.55	7.77 ± 1.23	15.34 ± 1.91
Deoxycholic	15.74 ± 1.37	20.64 ± 1.69	26.69 ± 1.17	28.59 ± 1.76
Chenodeoxycholic	7.71 ± 0.80	8.66 ± 0.54	7.83 ± 0.33	8.08 ± 1.39
Hyodeoxycholic	50.34 ± 3.34	53.80 ± 2.85	47.15 ± 1.66	39.09 ± 2.92
Cholic	2.94 ± 0.89	2.07 ± 0.32	3.33 ± 0.82	2.10 ± 0.56
α-Muricholic	17.44 ± 9.25	9.34 ± 2.08	7.35 ± 1.56	5.09 ± 2.66
β-Muricholic	12.61 ± 11.09	2.27 ± 0.75	2.74 ± 0.72	1.98 ± 0.55
Other	2.04 ± 0.77	1.33 ± 0.72	1.11 ± 0.34	1.10 ± 0.56

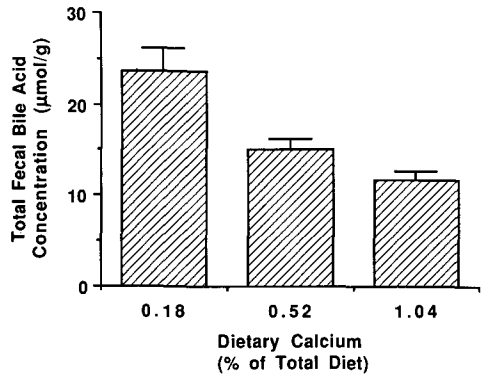

FIG. 4. Fecal bile acid concentration per dry fecal weight in relation to increasing dietary intake of calcium.

main mechanism of the increased bile acid excretion appears to be bile acid binding to fiber (39–42). In comparison to dietary fiber, dietary fat exerted a smaller, but significant influence on fecal bile acid excretion. The effect of fat on fecal bile acids appears to depend upon the type of fat in the diet (43,44). For example, the feeding of a high fat corn oil-based diet has been reported to be associated with an increased bile acid excretion, whereas *trans* fat in the diet had no significant effect (44). In contrast to dietary fiber and fat, calcium exerted no significant effect on bile acid excretion.

In contrast to our study, van de Meer *et al.* (45) found that supplemental dietary calcium increased the fecal bile acid excretion in healthy men. The authors speculated this to be due to the binding of calcium to intraluminal bile acids. The difference between our data and those by van der Meer *et al.* (45) seems to indicate that the rat model may not completely correlate with humans as far as calcium-bile acid interactions in the intestine are concerned.

Although fecal bile acid concentrations were not affected by either the fiber or fat content of the diet, they decreased significantly with increasing dietary calcium. The absence of an effect of wheat bran on fecal bile acid concentration appears to be in disagreement with reports by other investigators (45–47). However, although all these authors observed decreased bile acid concentrations, there were considerable variations in the total fecal bile acid excretion measurements. The increases in fecal bile acid concentration were associated with either a rise (46) or no change (45,47) in bile acid excretion. It appears, therefore, that the differences among the studies are mainly attributable to the varying degrees of dilution induced by dietary fiber. In the present study, both dietary fiber and calcium exerted a dilutional effect, as indicated by their close correlation with fecal wet as well as dry weight.

The absence, in this study, of a correlation between fecal bile acid concentrations and the amount of fat in the diet is in contrast to the results of most other authors who found that fecal bile acid concentrations increased with increasing dietary fat (7,19). The lack of a correlation in this study appears to be due to the finding that dietary fat had only a moderate effect on total fecal bile acid excretion and that there was no correlation between dietary fat and fecal weight. In addition, these findings may have been affected by the fact that in some of the other studies the dietary fat was considerably higher than in the present experiments (≥20% *vs.* ≤10%).

The decrease in fecal bile acid concentration, which we observed in response to increased dietary calcium, was the result of dilution, since fecal weight increased with dietary calcium without changes in total fecal bile acid excretion. Although calcium has been shown to bind to both free and micellar bile salts, it is not known whether this reaction influences the intestinal absorption of bile salts and calcium (28,48).

The fecal bile acid composition in this study was comparable to that reported by other authors (19,30,36,45,49). Similar to these reports, hyodeoxycholic and deoxycholic acids formed the major constituents of rat fecal bile acids. Among the minor bile acids, ω-muricholic acid has been observed by others to be present in rat feces (30,36,45). After completion of the present study, we reanalyzed eighteen fecal samples of different dietary fiber groups using an ω-muricholic acid standard recently made available to us through the courtesy of Dr. A.F. Hofmann, University of California, San Diego. Independent of the fiber content of the diet, less than 5% of the fecal bile acids consisted of ω-muricholic acid (data not presented). This observation was consistent with that of other investigators (30,36,45).

The finding that dietary fiber decreased the proportion of fecal bile acids consisting of hyodeoxycholic acid, but caused no significant change in the percent content of chenodeoxycholic acid, agrees with similar observations by others (8). However, in contrast to what others observed, the percentages of fecal lithocholic and deoxycholic acids increased with dietary fiber (8,16). The reason for the dietary fiber-related increase in intestinal bacterial 7-de-hydroxylation of bile acids is not clear. However, this

shift to secondary bile acids with increased cancer-promoting potential may not raise the cancer risk if the bile acids are not solubilized in the colon. Preliminary studies in our laboratory indicate that feeding of wheat bran to rats leads to a marked fall in both colonic pH and aqueous bile acid concentrations (51,52). The latter conditions may, therefore, reflect a decrease in the ability of bile acids to promote carcinogenesis in the colon, in spite of an overall increase in the proportion of lithocholic and deoxycholic acids in the feces.

In summary, the study shows that, in the rat, dietary wheat bran leads to increases in both total bile acid excretion and fecal weight, without changes in fecal bile acid concentration. The dietary fat increased the total fecal excretion but not the concentration of bile acids. In contrast, calcium feeding does not affect the total excretion of bile acids. However, since calcium causes an increase in fecal weight, it dilutes bile acids with a consequent decrease in their fecal concentration.

Studies are now in progress in our laboratories to determine the effect of the different diets on the solubility and watery concentrations of bile acids in the cecum. Such measurements, in conjunction with those obtained in the present study, should provide a possible explanation of the mechanisms by which dietary fiber and calcium may reduce the promotion of carcinogenesis by bile acids.

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Prolonged Retention of Doubly Labeled Phosphatidylcholine in Human Plasma and Erythrocytes After Oral Administration

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The plasma kinetics of a preparation of dilinoleoyl phosphatidylcholine (DLPC) specifically labeled with ^3H in the choline moiety and with ^{14}C in the 2-fatty acid (FA) were evaluated in six healthy volunteers after oral administration. Retention of both isotopes in plasma exceeded expectations, with a half-life in the elimination phase of 172.2 h for ^3H and 69.7 h for ^{14}C. Up to 60 d after administration, there were still significant levels of radioactivity present in plasma. The relative stability of the [^{14}C]FA label was demonstrated by the retention for more than 12 h of an isotope ratio close to that of the compound administered. The ^{14}C label of DLPC remained in position-2, as assessed by cleavage of plasma phospholipids with phospholipase A_2. The [^3H]choline label showed an early incorporation into high density lipoproteins and subsequently into low density lipoproteins (LDL); conversely, the ^{14}C radioactivity was rapidly incorporated into triacylglycerols that were mainly associated with very low density lipoproteins. Radioactivity measurements revealed that both isotopes remained the longest time in LDL. In red blood cell (RBC) lipids, [^3H]choline radioactivity accumulated over time, with a plateau after 48 h, whereas FA radioactivity accumulated more rapidly and was followed by a progressive decay. Analysis of the isotope ratio in these cells suggested an early incorporation of lyso products followed by rapid transfer of FA from plasma. The RBC maintained considerable radioactivity for a prolonged time, thus acting as a possible reservoir for the DLPC administered. Our study showed that dilinoleoyl PC remained in plasma longer than predicted based on earlier studies, and that after absorption the FA label was found in position 2.

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Administration of phosphatidylcholine (PC) is still being proposed in the therapeutic management of arterial disease (1,2) and nervous degenerative disease (3). Recent data have underlined the fact that PC liposomes may improve clearance of atherogenic lipoproteins, even in the absence of high affinity lipoprotein receptors in the liver (4,5). The still uncertain therapeutic role of exogenous PC in humans is partly due to a lack of studies that have examined in detail the metabolic fate of PC in humans. The few studies published have mostly used dilinoleoyl phosphatidylcholine (DLPC), labeled either in the choline or in the acyl moieties, or both (6–8). A general conclusion from these studies has been that PC is more than 90% absorbed from the intestine and that, while the choline moiety is retained in circulating PC, fatty acids (FA) are rapidly cleared (8). Animal experiments have suggested that PC may be hydrolyzed in the intestinal mucosa and then partly resynthesized (9,10). The remaining FA and glycerophosphate can then be utilized in triacylglycerol (TG) synthesis (10).

Studies on the distribution of orally administered PC in humans have shown a preferential association of PC with high-density lipoproteins (HDL) rather than with atherogenic lipoprotein particles (8). Inconsistent data, however, are on hand on the plasma elimination kinetics for the FA and choline moieties, with half-lives reported ranging from 32 h to 6 d (6–8). Earlier studies have generally not taken into account the possibility of a net transfer of phospholipids from lipoprotein particles to cells, which occurs *in vivo* (11), particularly in the lipid phase of cell membranes.

Kinetic studies of PC selectively labeled in the 2-position, where the fatty acid can be specifically cleaved by phospholipase A_2 (12), allow a more detailed evaluation of the enzymatic steps involved in the metabolism of the acyl moiety (13). This type of substrate was used in the present investigation, where the kinetics, distribution in plasma lipid and lipoprotein, as well as incorporation into red blood cells (RBC) of dilinoleoyl PC, labeled both in the choline and the 2-FA moieties, were evaluated in healthy volunteers. An additional objective of the study was to determine the metabolic stability of the PC molecule in plasma over nine long time periods after oral administration.

MATERIALS AND METHODS

Labeled PC. Doubly labeled dilinoleoyl PC (DLPC) was prepared by separately synthesizing 1-linoleoyl-2-[1-^{14}C]-linoleoyl-*sn*-glycero-3-phosphocholine ([^{14}C]DLPC) and 1,2-dilinoleoyl-*sn*-glycero-3-phospho-[*methyl*-^3H]choline ([^3H]DLPC) (14). The latter was prepared as follows: Linoleic acid, 5.34 mL (17.3 mmol), was first added to a solution of 1,1'-carbonyldiimidazole (17.3 mmol in 10 mL of dry $CHCl_3$) at room temperature, followed by warming for 1 h at 40°C. A solution of glycero-3-phospho-[*methyl*-^3H]choline (4.332 mmol, 3,387 μCi), prepared from 1,2-dipalmitoyl-*sn*-glycero-3-phospho-[*methyl*-^3H]choline (Amersham, Buckinghamshire, UK) by hydrolysis (14), and 2.631 mg (17.3 mmol) of 1.8-diazobicyclo[5.4.0.]undec-7-ene in 12 mL of dry $CHCl_3$ were added, and the resulting reaction mixture was kept at 40°C for 40 h. The mixture was then treated with 10 mL of methanol and stirred for 2 h at 40°C. Subsequently the mixture was cooled

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Abbreviations: AUC, area under the curve; BHT, butyl hydroxytoluene; CE, cholesteryl ester(s); Cl, clearance; DLPC, dilinoleoyl phosphatidylcholine, 1,2-linoleoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; FA, fatty acid(s); HDL, high density lipoprotein; GPC, glycero-3-phosphocholine; LCAT, lecithin-cholesterol acyltransferase; LDL, low density lipoprotein; LPC, lysophosphatidylcholine; LTP-I, II, lipid transfer protein-I, II systems; PC, phosphatidylcholine; PL, phospholipid(s); PLA_2, phospholipase A_2; RBC, red blood cells; TG, triglyceride(s); TL, total lipids; TLC, thin-layer chromatography; Vd_{ss}, volume of distribution at steady-state; VLDL, very low density lipoprotein.

to 0°C, and 10 mL of 20% HCl plus 10 mL of saturated aqueous NaCl was added. The organic layer was removed, and the aqueous layer was re-extracted with $CHCl_3$ (3 × 15 mL) and diethyl ether (2 × 10 mL). The organic extracts were dried over Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure. The crude product was purified by repeated flash chromatography (15) (CH_2Cl_2/MeOH, 1:1, vol/vol), giving 1050 mg (1.343 mmol, 1050 μCi) of 1,2-dilinoleoyl-*sn*-glycero-3-phospho-[*methyl*-^{3}H]choline in 31% yield.

In order to prepare DLPC labeled in the 2-position with [1-^{14}C]linoleic acid, a solution of [1-^{14}C]linoleic acid (1.058 mmol, 413 μCi) in 1.1 mL of dry $CHCl_3$ was added to a solution of 1.1′-carbonyldiimidazole (1.058 mmol) in 1.1 mL of dry $CHCl_3$. The mixture was warmed for 1 h at 40°C. Then solutions of 1-linoleoyl-*sn*-glycero-3-phosphocholine (LPC; 0.529 mmol), which was prepared by phospholipase A_2 hydrolysis of 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (kindly supplied by Nattermann GmbH, Cologne, Germany), and of 1.8-diazabicyclo[5.4.0]undec-7-ene (1.058 mmol), both in 1.1 mL of $CHCl_3$ were added. After stirring for 30 h at 40°C, the crude product was purified by repeated flash chromatography (CH_2Cl_2/MeOH, 1:1, vol/vol) (15), resulting in 302 mg (0.386 mmol, 302 μCi) of 1-linoleoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine ([^{14}C]DLPC) in 73% yield.

The final, doubly labeled product was obtained by dissolving in 50 mL of $CHCl_3$, 780 mg (780 μCi) of [^{3}H]DLPC, 260 mg (260 μCi) of [^{14}C]DLPC, and 5440 mg of unlabeled 1,2-dilinoleoyl phosphatidylcholine (DLPC, Nattermann). After solvent evaporation, 6480 mg of doubly labeled product (^{3}H: 0.1120 μCi/mg; ^{14}C: 0.0401 μCi/mg) was obtained and divided into 24 capsules, each containing 250 mg of lipid, with specific activities of 94 μCi/mmole and 31 μCi/mmole for ^{3}H and ^{14}C, respectively.

Experimental design. Six healthy subjects volunteered for the study (3 males and 3 females; age range, 24–44 yr) (Table 1). The subjects were fully informed of the modalities and goals of the study and signed a written consent form. None of the volunteers was undergoing treatment with drugs or was on a diet affecting plasma lipids or intermediary metabolism. Women were not on oral contraceptives. All subjects were found to have normal liver, kidney and bone marrow function. On day 0, after overnight fasting, each subject took three capsules of the labeled PC preparation, containing a total of 90 μCi of [^{3}H]DLPC and 30 μCi of [^{14}C]DLPC. Immediately after taking the capsules, each volunteer consumed a small breakfast consisting of whole milk, 200 mL; dry cookies, 50 g; and sugar, 10 g (calculated composition: 11.9% protein, 30.8% fat, 57.3% carbohydrates; 329.7 Kcal; 22.2% saturated FA; 8.2% monounsaturated FA; traces of polyunsaturated FA; cholesterol, 50.4 mg). Blood samples were drawn at time 0, and at 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96 and 120 h after DLPC administration. Samples drawn at the beginning of the day (24,48, 72, 96 and 120 h) were from fasting subjects. The design of this protocol was based on a previous study by Zierenberg and Grundy (8). Since analysis of the data revealed that the radioactivity was present in the circulation far longer than predicted, a further sample was collected from all subjects 60 d after administration (see below).

Urine was collected in a beaker, in the presence of sodium azide, at 0–2, 2–4, 4–8, 8–12, 12–24, 24–48, 48–72, 73–96, 96–120 h post-dose, and stored at −25°C until radioactivity was determined. Fecal excretion of radioactivity was also monitored by collecting daily fecal samples from all subjects. The samples were pooled in 5-d pools and later extracted for measuring radioactivity.

Analytical procedures for plasma and RBC. A minimum sample of 10 mL of blood was drawn [in Na_2 ethylenediaminetetraacetic acid (EDTA), 1 mg/mL] each interval in order to monitor plasma radioactivity (both ^{3}H and ^{14}C). In addition, at 2, 6, 12, 24, 48 and 120 h, an additional 20 mL of blood were drawn. These samples were subjected to a complete lipoprotein fractionation [very low density lipoprotein (VLDL), low density lipoprotein (LDL) and HDL] by standard density ultracentrifugation (16). In all samples, the cholesterol content was measured using a standard enzymatic method (17). Radioactivity distribution in plasma lipids (triglycerides, cholesteryl esters, phospholipids) was determined after thin-layer chromatography (TLC) of the lipid extract (see below). In order to monitor the stability of the choline and linoleoyl residues in PC, the specific activity of choline lipids and of lipid classes labeled in the acyl moieties was evaluated. At each interval, RBC were separated from the blood samples using ACD (citric acid, 71 mM; Na citrate, 85 mM; dextrose, 3 mM) as anticoagulant. After removal of platelet-rich plasma (PRP) by low-speed centrifugation, RBC were obtained after three cycles of centrifugation at higher speed (800 × g for 15 min, three times). Pellets were washed in ACD, and membranes were obtained by a final osmotic shock, followed by further washing and recentrifugation in distilled water.

Lipid extraction. Lipids in 1-mL aliquots of plasma and in lipoprotein fractions were extracted by stepwise addition of water, methanol and chloroform (2, 8, and 16 mL, respectively). This was followed by phase separation and

TABLE 1

Age, Sex, Physical Characteristics and Plasma Lipid Levels of Participating Volunteers[a]

Initials	Age (yr)	Sex	Weight (kg)	Height (cm)	Total chol. (mg/dL)	Triglycerides (mg/dL)	HDL chol. (mg/dL)	Phospholipids (mg/dL)
GG	44	F	53	158	190	51	72	237
FD	35	M	80	178	200	113	38	259
VV	30	F	47	158	155	34	75	231
BM	25	M	53	168	152	116	36	177
BL	24	M	56	168	128	66	38	180
EB	28	F	77	165	225	100	53	235

[a]Chol., cholesterol; HDL, high density lipoprotein.

collection of the organic layer. No radioactivity was detected in the aqueous layer. Lipids were extracted from RBC membrane pellets with solvents containing butylhydroxytoluene (BHT) (5 μg/mL) as antioxidant (18).

Chromatographic techniques. Cholesteryl esters (CD), TG and phospholipids (PL) in plasma lipid extracts were separated by TLC using hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol) as developing solvent. Separation of major plasma phospholipid classes was achieved by one-dimensional TLC using chloroform/methanol/aqueous ammonia (65:35:5, vol/vol/vol). Separation of RBC membrane lipids into individual phospholipid classes was accomplished by two-dimensional TLC (19). Authentic reference compounds (Supelco, Bellefonte, PA) were used for the identification of the products separated by TLC; chromatographic fractions were detected by exposure to iodine vapors.

Radioactivity determination. The amounts of ^{3}H and ^{14}C in aliquots of lipid extracts were assessed after evaporation of solvent in the presence of scintillation fluids using a Kontron scintillation counter with automatic quenching correction. The radioactivity in individual lipid classes separated by TLC was measured after scraping the respective silica fractions off the plates into counting vials. Counting efficiencies were corrected by submitting the labeled, original DLPC to the same chromatographic procedures. Aliquots of at least 1–2 mg of lipids were used for chromatographic separations and radioactivity counting. Final values are expressed as dpm/mg total lipid.

Pharmacokinetic analyses. These were done according to a two-pool model previously suggested by Wagener (6) and by means of a computerized procedure (20).

Localization of the ^{14}C label in DLPC. The position of the ^{14}C label in the compound administered and in the lipids extracted from plasma was verified by phospholipase A_2 (EC 3.1.1.4 from Naja naja naja venom, Sigma, St. Louis, MO) degradation according to Deems and Dennis (12). Briefly, the respective phospholipid fraction was subjected to enzymatic treatment, and the products were extracted and chromatographed by TLC. The LPC formed contained only ^{3}H; no ^{14}C radioactivity was found in the product indicating that the ^{14}C label was incorporated only in position-2 both of the original compound and the PC isolated from plasma. Tritium-labeled choline was detected in both the LPC and PC fractions.

RESULTS

In this study, as in a previous one (8) with a similar protocol, intestinal absorption of doubly labeled dilinoleoylphosphatidylcholine was found to be nearly complete as judged by measuring the area under the curve (AUC) of radioactivity in plasma over time (Table 2).

Absorption of the FA moiety was more rapid (Fig. 1), without reaching a plateau, while choline radioactivity rose steadily in the first 24 h after administration. The major kinetic parameters of the two isotopes are given in Table 2. Plasma half-lives ($t_{1/2}$) of the distribution (α) and elimination (β) phases, as calculated according to a two-pool model (6,8), indicate a more rapid distribution and elimination of the ^{14}C isotope, the volumes of distribution at steady-state (Vd_{ss}) being nearly identical for ^{3}H and ^{14}C. Calculation of absorption as: AUC = Dose/Clearance (Cl) yielded, for both isotopes, values

TABLE 2

Major Plasma Kinetic Parameters After Oral Administration of Doubly Labeled Dilinoleoyl Phosphatidylcholine to Healthy Volunteers (n = 6; X ± SEM)[a]

	^{3}H	^{14}C
Dose (dpm)	198 × 10^6	66 × 10^6
AUC (dpm/mL/h)	2,435,445 ± 79,719	323,664 ± 24,423
$t_{1/2}$ α (h)	21.17 ± 2.45	12.48 ± 2.70
$t_{1/2}$ β (h)	172.20 ± 8.01	69.65 ± 10.35
VD_{ss} (L)	20.33 ± 0.93	18.67 ± 1.21
T Cl (mL/h)	82.18 ± 2.69	210.98 ± 15.53

[a]AUC, area under the plasma concentration curves over time; $t_{1/2}$, half-life in the α and β phases; Vd_{ss}, volume of distribution at steady-state in liters (L); T Cl, total clearance. Absorption can be calculated from AUC = dose/Cl.

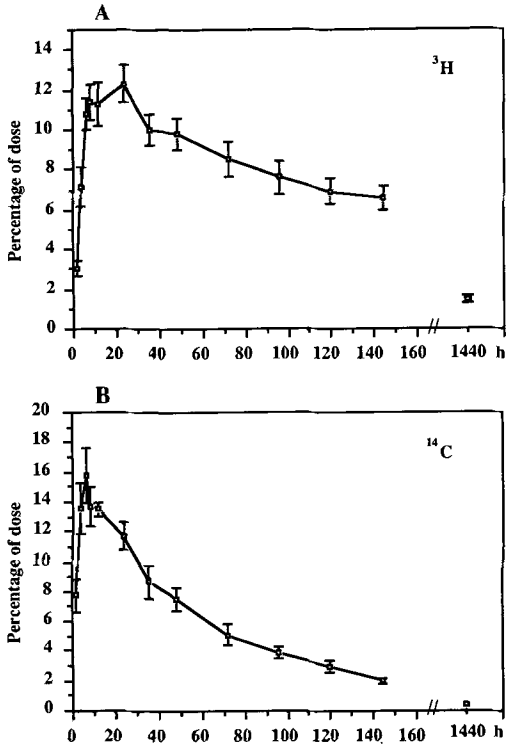

FIG. 1. Levels of ^{14}C and ^{3}H label in plasma after oral administration of doubly labeled dilinoleoyl phosphatidylcholine. Values are expressed as percentages of the administered dose. Means ± SEM, n = 6.

exceeding 95%. After 120 h from administration, *i.e.* at the projected end of the kinetic study, about 50% of the ^{3}H concentration at peak time was still present in plasma. For this reason, the volunteers were re-examined 60 d later (at 1440 h), when a low level of ^{14}C radioactivity (0.43 ± 0.1% of the dose) was still detected as well as a somewhat higher amount of ^{3}H (1.52 ± 0.1% of the dose). Insignificant amounts of radioactivity (≤5% for both isotopes) were found in urine and feces after 120 h.

The ratio of the two isotopes ($^{3}H/^{14}C$) in plasma (Fig. 2), at the earliest time periods (2–7 h after DLPC

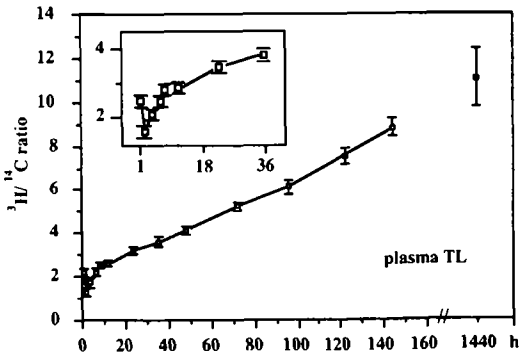

FIG. 2. $^3H/^{14}C$ ratios for total lipids in plasma. Means ± SEM, n = 6.

intake) fell below 3, *i.e.* below the isotope ratio originally present in the compound administered. The data indicate that the lyso compound and/or free glycerophosphocholine are absorbed or distributed at a different rate than are the fatty acids. PC isolated from plasma retained a $^3H/^{14}C$ ratio comparable to that of the compound administered up to 40 h after administration.

The ^{14}C isotope was retained in position-2 of the DLPC isolated from plasma at the different time intervals; in fact, no ^{14}C radioactivity was found in LPC after selective cleavage with phospholipase A_2 (PLA_2); also no ^3H label was detected in glycerophosphate (data not shown) (Table 3). These data, however, do not rule out the possibilities that (i) the fatty acid in position-2 was hydrolyzed and then reesterified in the same position, and (ii) that position-1 may at the end have contained fatty acids other than linoleic acid.

Distribution of labels in lipids and lipoprotein. The fate of the choline and FA moieties was somewhat different in terms of the distribution of the isotopes among the lipoprotein fractions (Fig. 3). Incorporation of ^3H radioactivity (Fig. 3, panels A-C) into VLDL was somewhat delayed and did not correlate with the incorporation of radioactivity into other lipoproteins. The FA radioactivity appeared, instead, almost immediately in VLDL, later in LDL and, particularly, HDL (panels D-F). The incorporation of both isotopes into LDL was progressive, reaching a maximum at 120 h, whereas the time courses of ^3H and ^{14}C radioactivity in HDL followed the same pattern as it did in plasma. The patterns of $^3H/^{14}C$ isotope ratios in the different lipoproteins over time (not shown) indicated that [^3H]choline was retained longer than the FA label. The VLDL $^3H/^{14}C$ ratio rose in fact constantly, from a value of about 0.2 to a value of 9 at 120 h, indicating that the progressive loss of ^{14}C label over time gave rise to particles relatively enriched in ^3H. A similar pattern was observed in HDL, with a rise of the ratio up to a value of about 6.5, whereas in LDL the ratios of the choline and FA radioactivities did not differ appreciably for the entire period under investigation from the $^3H/^{14}C$ ratio in the DLPC administered.

The distribution of the ^3H label among lipoprotein fractions (Fig. 4, panel A) changed over time: from a value of 47% in HDL, 35% in LDL, and 18% in VLDL and chylomicrons at the earliest time period up to about 60% in HDL, with a concomitant decline to 8% in VLDL at 16 h, and then remaining constant at around 10% thereafter. Analyses of the incorporation of [^3H]choline into individual lipid classes of plasma revealed that, as expected, [^3H]choline incorporation did not occur into lipid classes (*e.g.*, triglycerides, cholesteryl esters) other than phospholipids with incorporation of label into PC ranging from 73% at 4 h to 65% at 120 h (not shown) with respect to that in total PL (Table 4).

The percentage distribution of the ^{14}C label in lipoproteins (Fig. 4, panel B) showed a marked early incorporation into VLDL, followed by a rapid decline, consistent with the rapid catabolism of these lipoproteins (19). The isotope was readily incorporated into HDL and progressively into LDL. This metabolic behavior was confirmed by measuring the percentage distribution of the ^{14}C isotope into the different lipid classes of the three major lipoproteins (Fig. 5). Whereas the radioactivity in VLDL was found mainly in TG, [^{14}C]PC were the major labeled components of HDL and LDL. In both, PL radioactivity tended to decline slowly, with a progressive rise of CE labeling, particularly in LDL.

The $^3H/^{14}C$ ratios in plasma total PL and PC (Fig. 6) rose progressively, from an initial value of around 3 up to values of 30 and 40, respectively, which are far higher than those observed in total plasma lipids (Fig. 2). The faster increase of this ratio in total PL, as compared to that in PC, is due to the presence of LPC (containing only ^3H) in the former.

In RBC, a progressive accumulation of ^3H radioactivity in total lipids (TL) occurred over time, with a plateau

TABLE 3

Distribution of Radioactivity in the Hydrolysis Products of Plasma Lipids After Treatment with Phospholipase A_2 at Various Time Intervals After DLPC Administration[a]

	6 h		12 h		48 h	
Labels[b]	^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
Free fatty acids	—	717	—	509	—	184
Lysophosphatidylcholine	1,750	—	1,566	—	1,111	—
$^3H/^{14}C$ ratios between the hydrolysis products	2.4		3.1		6.0	
$^3H/^{14}C$ ratios of plasma	2.9		3.3		5.2	

[a]DLPC, dilinoleoyl phosphatidylcholine, 1,2-linoleoyl-*sn*-glycero-3-phosphocholine.
[b]Values are dpm in lipids extracted from 1.2 mL of plasma.

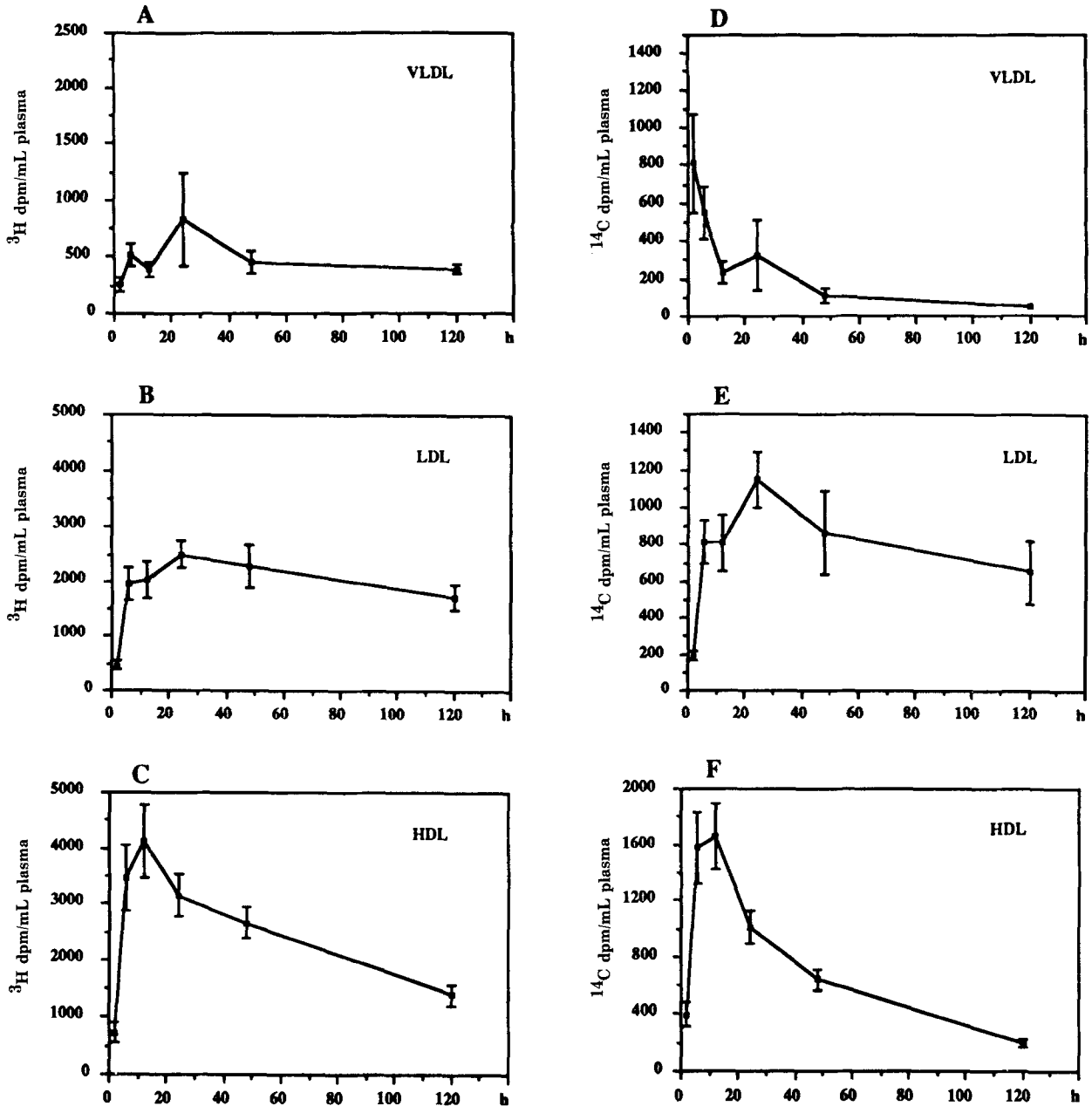

FIG. 3. ^{3}H (left) and ^{14}C (right) incorporation into lipoproteins after administering doubly labeled phosphatidylcholine. Values are dpm/mL of plasma. Means ± SEM.

TABLE 4

^{3}H Incorporation (dpm/mg of total lipids) into Plasma Phospholipids at Different Time Points[a]

Lipids	Time (h)			
	4	12	48	120
Phospholipids	214 ± 44.6	935 ± 115	697 ± 64	396 ± 32
Phosphatidylcholine	159 ± 31	749 ± 120	407 ± 28	258 ± 19
Lysophosphatidylcholine	59 ± 8	110 ± 17	66 ± 5	49 ± 3

[a]Values are means ± SE of six subjects.

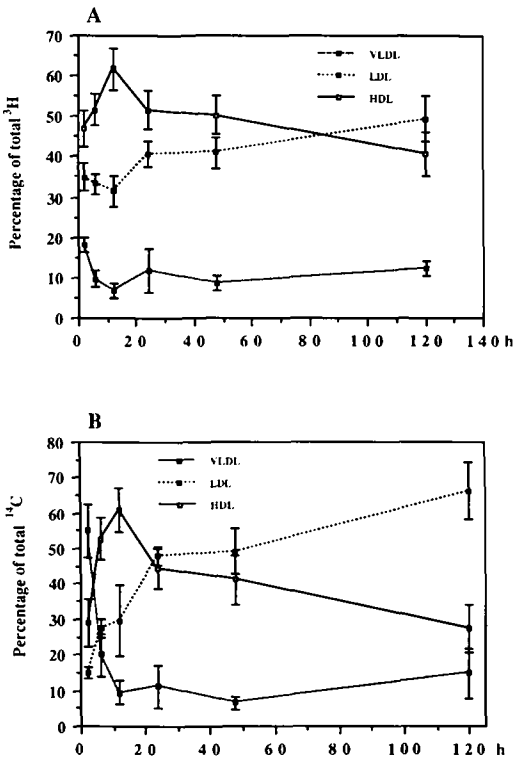

FIG. 4. ^{3}H (panel A) and ^{14}C (panel B) incorporation as percentage of total radioactivity into various plasma lipoproteins. Means ± SEM.

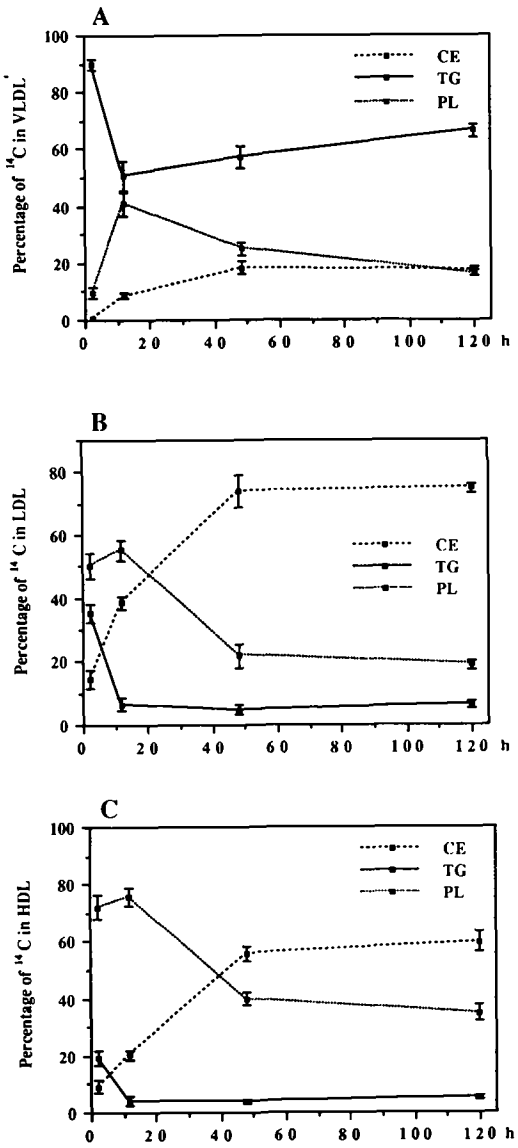

FIG. 5. Percentage distribution of ^{14}C radioactivity in different lipid classes of the three major lipoproteins. Means ± SEM.

after 48 h (Fig. 7, panel A). Values are expressed as dpm/mg of total lipids, corresponding to about 2.2×10^9 cells, as determined after the extraction of lipids from a known number of cells. The accumulation of ^{3}H in PC (panel B), measured as dpm in the fraction per mg of total RBC lipids, followed up to 48 h a trend similar to that in TL and in PL (not shown). A significant incorporation, progressively increasing with time up to 120 h, was detected in LPC (panel C).

The accumulation of the ^{14}C label in RBC total lipids (Fig. 7, panel D) was far more rapid, with a peak at 12 h, followed by a progressive decline, similar to that observed for ^{14}C in whole plasma. Incorporation of ^{14}C label in PC (not shown) followed a pattern which was superimposable over that of FA in total lipids. Accumulation of both isotopes in RBC occurred mainly in PL with a pattern very similar to that observed in total lipids (data not shown). Both isotopes essentially plateaued after 48 h, while radioactivity was still detectable 60 d after administration. The observed initial ^{3}H/^{14}C ratio in RBC lipids, that was far higher (about 3.7) than in plasma lipids (about 1.3) (Fig. 2) and in the administered DLPC, suggests an early incorporation of lyso products into RBC membranes.

DISCUSSION

The fate of orally administered PC has previously been investigated in experimental animals (9,10,21,22) and in humans (6–8). Only a small portion of PC is taken up as intact molecule and up to 50% of the LPC formed is re-esterified after absorption (8). The final water-soluble products, including *sn*-glycero-3-phosphocholine, glycerophosphate, glycerol and LPC can be absorbed *via* the portal route (10); LPC can also be reacylated in the mucosal cells by acyltransferases (22).

All information available on the fate of PC in humans, in particular of dilinoleoyl PC, has been derived from orally administered, randomly labeled PC species with FA label being associated with both the 1 and 2 positions (6–8). The labeled FA can readily be cleaved by various lipase-type activities at the absorption site or in the circulation (23). Fatty acids cleaved from position-2 are utilized for cholesterol esterification by way of lecithin-cholesterol acyltransferase (LCAT) (24), as well as for TG synthesis (10). Dilution with unlabeled linoleic acid is likely to occur at the 2 position, when the LPC produced is reacylated. However, no quantitative data are available to estimate the rate of this replacement. In previous investigations,

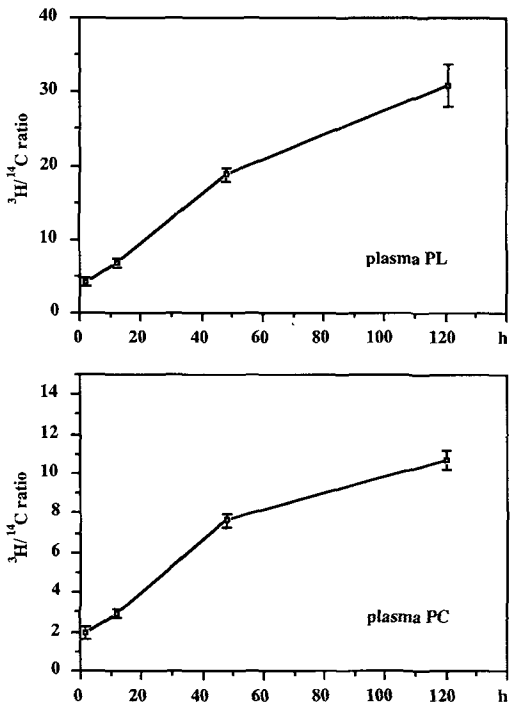

FIG. 6. $^3H/^{14}C$ ratios for plasma phospholipids (PL) and phosphatidylcholine (PC). Means ± SEM.

the extent and site of fatty acid hydrolysis/re-esterification could not be defined. Changes in $^3H/^{14}C$ ratio in blood following administration of DLPC labeled in both the 1 and 2 acyl moieties could result from FA loss from either position. After an oral dose of such a substrate, the $^3H/^{14}C$ ratios rose for several hours (8). Animal experiments, by contrast, strongly suggest that hydrolysis at the 2 position occurs preferentially in the intestinal lumen, due to the selective activity of pancreatic phospholipase A_2 (10,21,25).

In the present study, it was observed that the radioactivity in plasma maintained for many hours a $^3H/^{14}C$ ratio close to that of the product administered, an indication that the labeled FA remained in position-2 far longer than has been described for the 1 and 2 positions combined in previous studies (6–8). We did not determine, however, whether linoleate was retained in the 1 position.

The stability of the molecule was particularly evident in LDL. The exclusive association of FA radioactivity with the 2 position, as determined by selective hydrolysis with PLA_2, does not exclude the removal of the labeled FA; it indicates, however, that the ^{14}C label upon hydrolysis was not reintroduced into the 1 position. The data are therefore consistent with either a lack of hydrolysis in position-2 during intestinal absorption, contrary to what was found in animal experiments (10,21,22,25), or with hydrolysis followed by re-incorporation of the isotope-labeled FA into the same position. In view of the very low endogenous pool of free linoleic acid in the intestinal mucosal cells (26), recycling of the exogenous labeled FA cannot be excluded. In the present study, however, some incorporation of [^{14}C]FA released from PC into TG did occur as clearly shown by the relatively high proportion of total ^{14}C radioactivity in VLDL-TG. Recently, Ikeda *et al.* (27) suggested that more than 80% of PC, recovered from the intestinal lymph of rats after oral administration of the product labeled in the choline moiety, was still intact.

Since an earlier study using PC labeled in the 1 and 2 FA moieties found a relatively rapid decline of FA associated radioactivity both in plasma and in RBC (8), our experiment was originally set up for 144 h only. Surprisingly, ^3H and ^{14}C radioactivity was retained in plasma lipids far longer than predicted, and an appreciable

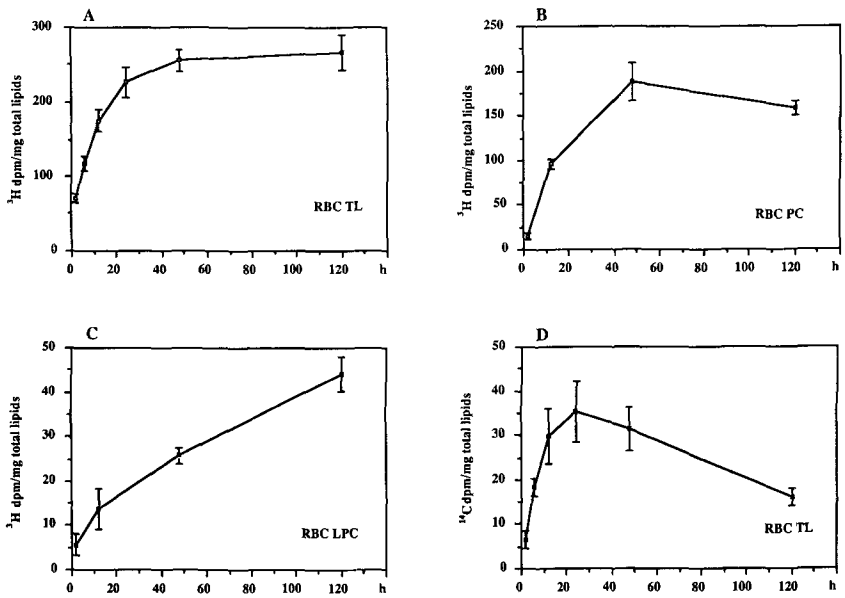

FIG. 7. ^3H and ^{14}C incorporation into total lipids of red blood cells (RBC), expressed as dpm/mg of total lipids (TL) over time. means ± SEM.

amount of labeling was found even 60 d after administration.

The fate of absorbed PC can be related to the incorporation into selected lipoproteins, followed by catabolism in plasma (28), or to transfer/exchange processes mediated in plasma by the lipid transfer protein-I and II (LTP-I and II) systems (29). The rapid incorporation of ^{14}C into VLDL-TG suggests an early partial degradation of PC. HDL ^{14}C-labeled PL showed a slow disappearance, with a concomitant rise in [^{14}C]CE, resulting from cholesterol esterification *via* LCAT; transfer of HDL cholesterol to LDL would then be accomplished by LTP-I (30).

The ^{14}C distribution pattern in the different lipid classes of the three major lipoproteins (Fig. 5) supports the hypothesis of a transfer of surface PL from small chylomicrons to HDL, which show the highest relative [^{14}C]PL radioactivity. The behavior of the isotopes in LDL and HDL does not seem to bear a close relationship with that of VLDL associated radioactivity. In LDL, both ^{14}C and ^{3}H decline slowly, with a stable $^{3}H/^{14}C$ ratio, very close to that of the compound administered, thus supporting the hypothesis by Williams *et al.* (5) that PC may constitute a relatively stable portion of these lipoproteins. About 50% of the total ^{3}H radioactivity at 120 h was associated with the LDL fraction and, in this fraction, the $^{3}H/^{14}C$ ratio was the same as in the original PC. Calculations of the relative specific activity of PC in LDL *vs.* that of the PC administered indicate that exogenous PC constituted about 1.5% of LDL-PC at the peak plasma concentration, after a single oral dose of 750 mg.

Finally, radioactivity was progressively incorporated into RBC lipids, with choline radioactivity still plateauing 120 h after administration. The $^{3}H/^{14}C$ ratios in RBC rose at a much slower rate than in plasma lipids for the first 40 h, thus suggesting minimal FA hydrolysis.

Taken together, the observations support the conclusion that dilinoleoyl PC is retained in plasma unchanged for a substantial period of time. However, some hydrolysis gives rise to the transfer of LPC to RBC membranes, while most of the intact molecule tends to become associated with LDL. At the earliest time points, a portion of ^{14}C radioactivity was also present in VLDL-TG. The observed long-lasting association of PC with LDL is of interest because of the well-known role of LDL in cellular cholesterol homeostasis and in the receptor mediated delivery of polyunsaturated fatty acids to cells (31), findings which tend to support the early hypothesis by Friedman *et al.* (32) that PC is not just a short-lived circulating molecule.

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Reduction in Triacylglycerol Levels by Fish Oil Correlates with Free Fatty Acid Levels in *ad libitum* Fed Rats

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Rats were fed (for 2 or 6 wk) purified diets containing lard (LD) or menhaden oil (MO) at two levels of dietary fat, *i.e.*, at 11.5 and 20.8% of energy in the low fat (LF) and the medium fat (MF) diets, respectively. Following the diet period, rats were sacrificed after either an overnight fast or after uninterrupted *ad libitum* feeding. The studies were designed to investigate the dependence of our previously reported effects of MO, *i.e.* the reduction of plasma free fatty acid (FFA) levels and accumulation of hepatic triacylglycerols, on the dietary fat concentration and the nutritional state of the animal at the time of sacrifice. Reductions in plasma triacylglycerol and cholesterol levels in MO-fed relative to LD-fed rats were observed under all conditions. FFA levels were consistently reduced by MO-feeding at both dietary fat concentrations, but only when blood was sampled from *ad libitum* fed rats. Under these conditions there was a significant positive relationship between plasma FFA and triacylglycerol concentrations. Reduction in plasma FFA levels may be an additional mechanism associated with the triacylglycerol-lowering effect of fish oil (FO). The LF and MF MO diets caused a rise in plasma glucose levels with no significant change in insulin concentration, indicating that the reduction of FFA by MO was not related to changes in insulin concentration or insulin sensitivity. The MO diets had no effect on skeletal muscle or epididymal adipose tissue lipoprotein lipase activity, demonstrating that catabolism of triacylglycerol-rich lipoproteins contributes little, if any, to the MO-dependent reductions of plasma triacylglycerol and FFA. The previously reported accumulation of hepatic triacylglycerols after high fat (HF; 30% of energy) MO-feeding was not observed with the LF or MF MO diets, suggesting that the apparent direct inhibition of triacylglycerol secretion by FO imposes a rate-limitation only when feeding HF diets.
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In earlier studies (1,2) we observed significant but inconsistent reductions in plasma free fatty acid (FFA) levels in fish oil (FO)-fed, overnight fasted rats. An effect on plasma FFA by FO was also observed by Singer *et al.* (3), who reported that dietary ω3 polyunsaturated fatty acid (n-3 fatty acids) supplementation in hyperlipidemic patients resulted in a marked decrease in FFA during a standard glucose tolerance test that was associated with a decline in serum triacylglycerol levels. The authors (3) suggested that the decrease in FFA indicated reduced lipolysis and that it might contribute to the triacylglycerol-lowering effect of n-3 fatty acids. We and others also demonstrated a decrease in adipose tissue mass in FO-fed rats (2,4,5) that was accompanied by a decrease in adipocyte volume without a decrease in fat cell number (4,5). Additionally, we found that this decrease in adipose tissue mass was positively correlated with the FO-mediated changes in plasma FFA (2), suggesting a causal relationship. Since a reduction of plasma FFA may reflect an important mechanism involved in FO-mediated effects, we investigated why this effect of FO has not been routinely observed in the numerous studies conducted with dietary FO. Specifically, we considered whether observing a reduction in plasma FFA levels was dependent upon the concentration of dietary n-3 fatty acids or the nutritional state (overnight fasted *vs. ad libitum* fed rats) of the animals at the time of sacrifice. We also investigated the relationship between plasma FFA and the lowering of plasma triacylglycerol levels by FO. A preliminary account of this work has been presented (6).

MATERIALS AND METHODS

Experimental design and diets. Male Sprague-Dawley rats (225–250 g; VAF/Plus, Charles River Laboratories, Inc., Raleigh, NC) were housed in suspended transparent polycarbonate cages with stainless steel wire bottoms in an animal facility maintained at 22 ± 1°C, 70% humidity and with a 12-h light/dark cycle. Rats were acclimated to the surroundings and fed a standard AIN-76A purified diet (7) (Table 1, low fat diet with corn oil as the fat source; Research Diets, Inc., New Brunswick, NJ) for 1 wk. Animals had free access to deionized water.

Following the acclimation period, rats were matched for weight and assigned to respective groups (6 rats/group), based on the diet, the length of feeding, and the nutritional state of the animal at sacrifice. Rats were assigned to one of four diet groups. These included low fat (LF; 11.5% of energy) and medium fat (MF; 20.8% of energy) diets, prepared with either lard (LD) or menhaden oil (MO; vacuum stripped MO provided by the National Institutes of Health, Bethesda, MD) as the major fat source (Tables 1 and 2). All diets contained a minimum of 4% of energy from corn oil (CO) to prevent essential fatty acid deficiency. Thus, LD and MO provided 7.5 and 16.8% of energy in the LF and MF diets, respectively. The purified diets were based on the AIN-76A diet (7) and contained identical ingredients except for the source of fat. In the MF diets, the additional fat energy was balanced by an isocaloric reduction in carbohydrate (sucrose/corn starch, 2:1, w/w). Therefore, the nutrient (protein, vitamins and minerals)/energy (g/kJ) ratios were the same in all diets. As in our previous studies (1,2), the diets were also balanced for cholesterol (8.96 mg/1000 kJ) and β-sitosterol (76.8 mg/1000 kJ) relative to the energy content of the diets, and for the antioxidant content (α- and γ-tocopherols, and *tert*-butylhydroquinone; 1.5, 1.2 and 0.2 mg/g fat) relative to the amount of fat in the diets. The importance of including antioxidants in the diets is emphasized by a recent report by Haglund *et al.* (8) indicating that an FO diet rich in vitamin E (1.5 IU/g fat) had a greater triacylglycerol-lowering effect than one with only 0.3 IU/g. Our diets

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Abbreviations: CO, corn oil; FFA, free fatty acids; FO, fish oil; HF, high fat; LD, lard; LF, low fat; LPL, lipoprotein lipase; MF, medium fat; MO, menhaden oil.

TABLE 1

Composition of Purified Diets[a]

Ingredients	Low fat g/kg	Low fat Energy (%)	Medium fat g/kg	Medium fat Energy (%)
Fat[b]	50.0	11.5	95.0	20.8
Sucrose/corn starch (2:1, w/w)	650.0	66.6	589.0	57.3
Casein (alcohol extracted)	200.0	20.5	211.0	20.5
Cellulose	50.0	0.0	52.6	0.0
DL-Methionine	3.0	0.0	3.2	0.0
Salt mix[c]	35.0	0.4[d]	36.8	0.4[d]
Vitamin mix[e]	10.0	1.0[d]	10.5	1.0[d]
Choline bitartrate	2.0	0.0	2.1	0.0

[a]Diets were based on the AIN-76A diet. Medium fat diets were prepared by isocaloric substitution of fat for carbohydrate; thus the nutrient (protein, vitamins and minerals)/kJ ratios were unchanged from the low fat diet. The energy contents of the respective diets were 16.4 and 17.3 kJ/g for the low and medium fat diets.

[b]All diets contained a minimum of 4% of energy from corn oil to prevent essential fatty acid deficiency. The remaining fat energy was supplied by either lard or menhaden oil. Thus, lard and menhaden oil provided 7.5 and 16.8% of energy in the low and medium fat diets, respectively. Menhaden oil was provided by the Fish Oil Test Materials Program of the National Institutes of Health.

[c]Salt mix (in g/kg of mixture) calcium phosphate, dibasic, 500; magnesium oxide, 24; potassium citrate, monohydrate, 220; potassium sulfate, 52; sodium chloride, 74; chromium potassium sulfate, 0.55; cupric carbonate, 0.3; potassium iodate, 0.01; ferric citrate, 6.0; manganous carbonate, 3.5; sodium selenite, 0.01; zinc carbonate, 1.6; sucrose, 118.03.

[d]As sucrose.

[e]Vitamin mix (in g/kg of mixture): Vitamin A palmitate (500,000 IU/g), 0.8; Vitamin D_3 (400,000 IU/g), 0.25; Vitamin E acetate (500 IU/g), 10.0; menadione sodium bisulfate, 0.08; biotin (1%), 2.0; cyanocobalamin (0.1%), 1.0; folic acid, 0.2; niacin, 3.0; calcium pantothenate, 1.6; pyridoxine HCl, 0.7; riboflavin, 0.6; thiamin HCl, 0.6; sucrose, 979.17.

TABLE 2

Fatty Acid Composition of Lard and Menhaden Oil

Fatty acid	% of Total fatty acids Lard	Menhaden oil
14:0	1.4	7.6
14:1	0.1	0.1
16:0	24.5	16.6
16:1n-9	3.3	0.2
16:1n-7	—	10.3
18:0	15.0	3.1
18:1n-9	42.8	8.1
18:2n-6	10.5	1.2
18:3n-3	1.0	1.2
20:1	0.6	2.3
20:5n-3	—	14.8
22:1n-9 + n-11	—	0.5
22:5n-3	—	2.5
22:6n-3	—	8.7
Saturated	40.9	27.3
Monounsaturated	46.8	21.5
Polyunsaturated		
n-6	10.5	1.2
n-3	1.0	27.2
P/S ratio	0.28	1.04

contained approximately 1.4 IU of vitamin E/g fat along with *tert*-butylhydroquinone. The MO diets were prepared in our laboratory by addition of MO to a basal diet as previously described (1,9). All other diets were custom-prepared by a commercial vendor (Research Diets, Inc.). Diet cups were placed in the cages such that the diets were not exposed to direct light. All diets were replaced every third or fourth day (9).

Rats were fed one of the four diets for 2 wk (12 rats/diet group) or 6 wk (6 rats/diet group). At the end of the feeding period, half of the animals from each group were sacrificed in the morning after uninterrupted *ad libitum* feeding. The other half were sacrificed after an overnight fast. The data from 6 wk fed rats are presented in the text only when relevant to the discussion.

Plasma and tissue collection. At the time of sacrifice, rats were anesthetized with pentobarbital (5 mg/100 g body wt). Blood was drawn from the abdominal aorta into a syringe containing 0.25 mL of 0.2 M ethylene diaminetetraacetic acid, pH 7.4, and plasma was collected by centrifugation at 500 × *g* (4°C) for 20 min. Immediately after drawing blood, livers were rapidly removed, weighed and freeze-clamped with aluminum tongs precooled in liquid nitrogen (10). The thin layer of frozen liver tissue was later ground into a fine powder under liquid nitrogen. Soleus muscles and epididymal fat pads were collected, weighed and immediately frozen by dropping into liquid nitrogen. All samples were stored at −80°C until assayed.

Analytical procedures. Plasma triacylglycerol, cholesterol and glucose were measured using Baker kits with the Encore Chemistry System centrifugal analyzer (Baker Instruments Corp., Allentown, PA). The assay for FFA (Amano International Enzyme kit reagents, Troy, VA) was adapted for use on the Encore chemistry System (1). Plasma insulin was determined by radioimmunoassay using a commercial kit (Ciba Corning Diagnostics Corp., Medfield, MA).

Liver triacylglycerol was extracted and assayed as previously reported (1). Soleus muscle and epididymal adipose tissue lipoprotein lipase (LPL) activities were measured as described earlier (2).

Statistical analysis. Statistical differences between LD and MO diet groups were determined by the Student's *t*-test. Correlation between measured parameters was determined by least squares regression analysis. All statistical analyses were performed utilizing the SYSTAT statistical software package (SYSTAT, Inc., Evanston, IL).

RESULTS AND DISCUSSION

Plasma triacylglycerol, cholesterol and free fatty acids. The reduction in plasma triacylglycerol and cholesterol levels in MO-fed relative to LD-fed rats was observed consistently under all conditions of dietary fat concentration and nutritional state (Table 3). In this study we considered whether the normal rise in plasma FFA that occurs with fasting might sometimes mask effects of FO on FFA, and thus, explain the inconsistencies of our previous observations with overnight fasted rats (1). After 2 wk of feeding, a significant decrease in plasma FFA was observed in the MO- *vs.* LD-*ad libitum* fed rats at both fat concentrations. In contrast, after an overnight fast, FFA were reduced

TABLE 3

Plasma Metabolites from *ad libitum* Fed and Overnight Fasted Rats[a]

Nutritional state	Diets: Fat concentration	Diets: Fat source	Triacylglycerols (mM)	Cholesterol (mM)	Free fatty acids (μM)	Glucose (mM)	Insulin (μU/mL)
ad libitum Fed	Low fat	LD	3.35 ± 0.11	2.40 ± 0.18	316 ± 35	8.9 ± 0.4	70 ± 8
		MO	1.25 ± 0.20[b]	1.86 ± 0.13[b]	207 ± 21[b]	10.4 ± 0.3[b]	65 ± 6
	Medium fat	LD	4.25 ± 0.29	2.45 ± 0.47	348 ± 34	9.0 ± 0.4	93 ± 9
		MO	1.74 ± 0.07[b]	1.47 ± 0.16[b]	168 ± 16[b]	9.9 ± 0.2[b]	75 ± 8
Overnight fasted	Low fat	LD	1.90 ± 0.21	1.78 ± 0.13	418 ± 41	8.1 ± 0.2	66 ± 6
		MO	0.69 ± 0.06[b]	1.60 ± 0.16	410 ± 36	8.7 ± 0.6	47 ± 8
	Medium fat	LD	1.44 ± 0.12	1.99 ± 0.18	499 ± 52	5.9 ± 0.3	56 ± 4
		MO	0.78 ± 0.06[b]	1.29 ± 0.08[b]	343 ± 18[b]	8.2 ± 0.7[b]	44 ± 3

[a]Rats were fed the respective diets for 2 wk before sacrifice. Values are the mean ± SE (n = 6). Abbreviations: MO, menhaden oil; LD, lard.
[b]Significant difference between MO- and LD-fed animals, $P < 0.05$.

only in the MF MO group. After 6 wk this effect was again significant in *ad libitum* fed rats (with LF diets, 429 ± 54 *vs.* 186 ± 23 μM FFA; and with MF diets, 338 ± 92 *vs.* 176 ± 21 in LD-fed and MO-fed rats, respectively) but not seen at all in the overnight fasted rats (with LF diets, 406 ± 13 *vs.* 410 ± 38; and with MF diets, 463 ± 45 *vs.* 413 ± 53 in LD-fed and MO-fed rats, respectively). In our earlier study (1) we made similar observations with high fat (HF) diets (30% of energy; identical to diets described in Table 1 with an isocaloric substitution of fat for carbohydrate). After uninterrupted *ad libitum* feeding of HF CO, LD and MO diets for 2 wk, FFA were significantly reduced in the MO-fed rats relative to the CO- and LD-fed rats (435 ± 34, 448 ± 68 and 201 ± 43 μM in CO-, LD- and MO-fed rats, respectively; $P < 0.05$; data from *ad libitum* fed rats were not previously reported). However, like the LF- and MF-fed rats after an overnight fast, there were no differences between the three HF diet groups (351 ± 28, 392 ± 102 and 377 ± 83 μM in CO-, LD- and MO-fed rats, respectively). Thus, the data clearly demonstrate that blood must be sampled in the fed state in order to consistently observe the effects of dietary FO on plasma FFA.

There was a highly significant correlation between plasma triacylglycerol and FFA levels in *ad libitum* fed rats (Fig. 1; data from Table 3). We also observed this with *ad libitum* fed rats receiving HF diets [r = 0.74, $P < 0.006$, n = 12; from the experiment of (1), but data not previously shown]. These data strongly suggest that along with other mechanisms, the lowering of plasma FFA levels by FO-feeding may contribute significantly to the decrease in plasma triacylglycerols by decreasing the supply of substrate for hepatic triacylglycerol synthesis. This is consistent with the recent report of Singer *et al.* (3) and focuses attention to possible effects of dietary FO on adipose tissue lipolysis, as has been observed by Parrish *et al.* (5).

Plasma insulin and glucose. We previously reported that feeding HF MO diets followed by an overnight fast resulted in a reduction in plasma insulin levels with an accompanying reduction or no change in plasma glucose levels (1,2). This is consistent with an improvement in peripheral insulin sensitivity. In the present experiment with the LF and MF MO diets, insulin levels were only

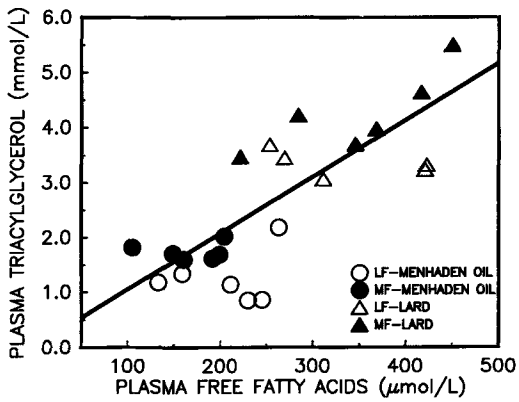

FIG. 1. Correlation between plasma free fatty acids and plasma triacylglycerols from *ad libitum* fed rats [data from Table 3; for all data, r = 0.71, $P < 0.001$, n = 23; for low fat (LF) diets, r = 0.68, $P = 0.022$, n = 11; and for medium fat (MF) diets, r = 0.92, $P < 0.001$, n = 12].

slightly lowered or unchanged relative to those in LD-fed rats (Table 3; approaching significance only in overnight fasted rats adapted to the LF or MF MO diets, $P = 0.074$ and 0.059, respectively). Under these conditions, plasma glucose was generally elevated (Table 3). The rise in glucose may have been in response to the slight reductions in plasma insulin levels, but it is also possible that insulin sensitivity (peripheral or hepatic) actually worsened with LF and MF MO-feeding. The data clearly show that the lower plasma FFA levels seen with MO-feeding at all concentrations cannot be explained by either an increase in insulin concentration or improved insulin sensitivity at the site of the adipose tissue.

LPL and adipose tissue mass. Unlike our earlier study (2) in which we observed a significant elevation of soleus muscle LPL in HF MO-fed rats, feeding LF and MF MO diets had no effect on soleus muscle or epididymal adipose tissue LPL activities from rats sacrificed in either the fed or overnight fasted state (data not shown). Since these diets did cause significant reductions in plasma triacylglycerol and FFA levels (Table 3), it is unlikely that regulation of these enzyme activities contributes significantly

TABLE 4

Liver Weight and Triacylglycerol from *ad libitum* Fed and Overnight Fasted Rats[a]

Nutritional state	Diets: Fat concentration	Diets: Fat source	Liver weight: (g)	Liver weight: /Body weight (× 100)	Liver triacylglycerols (μmol•liver^{-1})
ad libitum Fed	Low fat	LD	16.8 ± 0.7	4.3 ± 0.1	429 ± 61
		MO	16.4 ± 0.5	4.2 ± 0.1	259 ± 37[b]
	Medium fat	LD	17.9 ± 0.3	4.4 ± 0.1	673 ± 111
		MO	18.4 ± 0.4	4.6 ± 0.1[b]	411 ± 51[b]
Overnight fasted	Low fat	LD	13.0 ± 0.7	3.4 ± 0.1	250 ± 35
		MO	13.1 ± 0.8	3.4 ± 0.2	254 ± 37
	Medium fat	LD	11.3 ± 0.6	2.9 ± 0.1	342 ± 67
		MO	13.3 ± 0.4[b]	3.4 ± 0.1[b]	270 ± 33

[a]Rats were fed the respective diets for 2 wk before sacrifice. Values are the mean ± SE (n = 6). MO, menhaden oil; LD, lard.
[b]Significant difference between MO- and LD-fed animals, $P < 0.05$.

to either of these effects of FO, at least at the FO concentrations used in this study. Although not statistically significant, there was a tendency for the LF and MF MO-fed rats (both *ad libitum* fed and overnight fasted) to have reduced epididymal adipose tissue wt/body wt ratios (not shown) as observed previously by us (2; with HF diets) and others (4,5). In our earlier study we demonstrated that this reduction in adipose tissue mass was positively correlated with the FO-dependent decrease in FFA levels. It is, thus, reasonable to propose that the reduction in FFA is linked to the decrease in adipose tissue mass. Parrish *et al.* (5) reported that the decrease in adipose tissue mass was accompanied by a decrease in lipoprotein binding and basal lipolysis in epididymal adipocytes, and an increase in hormone-stimulated lipolysis in both epididymal and perirenal adipocytes. These effects together with the hypotriglyceridemic effect of FO could explain the decrease in adipose tissue mass and the related decrease in plasma FFA.

Liver weight and triacylglycerol levels. In our earlier study (1), we observed that feeding HF MO diets caused significant hepatomegaly that was due entirely to hypertrophy. The current study demonstrates that this effect of FO, which is reflected in an increase in the liver wt/body wt ratio was also observed in the MF, but not the LF diet groups regardless of the nutritional state of the rats at sacrifice (Table 4). We (1) and others (11) have suggested that the hepatomegaly is related to peroxisomal proliferation induced by FO-feeding.

We also reported earlier (1) that there was an elevation in liver triacylglycerols caused by HF MO-feeding concomitant with the lowering of plasma triacylglycerol levels, suggesting a primary inhibition of hepatic triacylglycerol secretion by MO, independent of triacylglycerol synthesis. Our suggestion is supported by the recent *in vitro* experiment by Lang and Davis (12) indicating that eicosapentaenoic acid and docosahexaenoic acid decreased the secretion of both triacylglycerols and apo B by cultured rat hepatocytes independent of their synthesis, but only at the highest concentration tested (1 mM fatty acid/0.13 mM albumin). Consistent with this, we did not see an accumulation of liver triacylglycerols after feeding rats LF or MF diets (Table 4). In fact, there was a significant reduction in liver triacylglycerol levels in MO-fed rats (Table 4; *ad libitum* fed only) under these dietary conditions, resulting in a highly significant relationship between the plasma and liver triacylglycerol concentrations (data from Tables 3 and 4; r = 0.71, $P < 0.001$, n = 24). Thus, our data suggest that very low density lipoprotein secretion *per se* may be rate-limiting, but only with HF diets when the supply of exogenous FFA may exceed the capacity of the liver to dispose of them. With the MF and LF diets, the rate of hepatic triacylglycerol synthesis appears to limit secretion.

Conclusion. The present study offers an explanation for the apparent variable response of plasma FFA to feeding dietary FO. Our data indicate that this is due to the nutritional state of the animal at the time of blood sampling. The FO-induced reduction of plasma FFA was consistently observed in *ad libitum* fed animals at all fat levels (LF, MF and HF), resulting in a strong positive relationship between plasma FFA and triacylglycerols. This strongly suggests that lowered plasma FFA levels by FO-feeding may play a significant role in the triacylglycerol-lowering effects by limiting the availability of substrate for hepatic triacylglycerol synthesis. We have shown that the reduction in FFA levels by MO-feeding was not related to changes in the insulin concentration or insulin sensitivity. The previously observed effect of the HF MO diet on skeletal muscle LPL was not seen with the LF or MF diets, suggesting that catabolism of triacylglycerol-rich lipoproteins contributes little, if any, to the FO-dependent reductions of plasma triacylglycerols or FFA. The current study indicates that the apparent direct inhibition of triacylglycerol secretion by FO (1) imposes a rate-limitation only when feeding HF diets. At the lower fat concentrations, hepatic triacylglycerol synthesis appears to limit secretion.

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Borage or Primrose Oil Added to Standardized Diets Are Equivalent Sources for γ-Linolenic Acid in Rats

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The aim of this study was to evaluate the effect of different doses and sources of dietary γ-linolenic acid (GLA) on the tissue phospholipid fatty acid composition. Rats fed four different levels of GLA (2.3, 4.6, 6.4 and 16.2 g of GLA/kg diet) in the form of either borage oil or evening primrose oil during 6 wk were compared with animals fed corn oil. The levels of dihomo-γ-linolenic acid (DHLA) and GLA showed a significant dose-related increase in liver, erythrocyte and aorta phospholipids. Moreover, the arachidonic acid/DHLA ratios in tissues decreased with increasing intake of dietary GLA. There was no significant difference in tissue GLA and DHLA levels within groups given equal amounts of dietary GLA either as borage oil or evening primrose oil. The amount of dietary GLA administered did not significantly influence prostaglandin E_2 production in stimulated aortic rings and thromboxane B_2 levels in serum; however, an increase in prostaglandin E_1 derived from DHLA was observed in the supernatants of stimulated aorta.
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γ-Linolenic acid (GLA), an essential fatty acid (EFA) of the n-6 series, is synthesized from dietary linoleic acid (LA) by Δ6 desaturation, which is rate-limiting, and then is rapidly elongated to dihomo-γ-linolenic acid (DHLA) (1). A breakdown in this regulation has been associated with various disease states, including diabetes, atopic eczema, premenstrual syndrome, and coronary heart disease (2–10). In fact, several clinical studies have demonstrated improvement in patients with atopic eczema after administration of GLA-rich oil (11–15). DHLA is the precursor of prostaglandin E_1 (PGE_1), which has potent vasodilatory, anti-aggregatory and anti-inflammatory properties (16–21). In addition, DHLA can be metabolized to 15-hydroxy-eicosatrienoic acid, a potent inhibitor of lipoxygenase (22–24). DHLA also competes with arachidonic acid (AA) in various oxidative processes catalyzed by lipoxygenases and cyclooxygenases and thus can ameliorate the potential deleterious effect of elevated proinflammatory AA metabolites. GLA and DHLA may therefore be useful to correct for abnormalities in EFA metabolism and/or imbalanced eicosanoid formation (2). Seeds of borage, evening primrose and black currant plants contain relatively large amounts of GLA. Several studies have shown that the application of GLA-rich oils results in the accumulation of GLA and its metabolite DHLA in tissue phospholipids (25–35). In previous studies, GLA-containing oils have been compared by feeding equal amounts of fat but different amounts of GLA (36,37). In one study, the amount of GLA was kept constant in all groups, but the total amount of fat varied between 1.6% of the diet (BO) to 4.5% of the diet (EPO); however, with GLA-containing oils being the only fat source, this led to considerable variations in linoleic acid levels in the diet (38).

The aim of the present study was to investigate the effect of the dose and source of GLA on the fatty acid composition of liver, erythrocytes and aorta and on prostanoid formation in stimulated aorta in animals fed equal amounts of fat and GLA.

MATERIALS AND METHODS

Animal treatment. Male albino SPF rats, weighing about 200 g and maintained on a standard pelleted diet, were randomly divided into seven groups. The rats were fed a fat-free diet, SODI2007, obtained from Klibamühle (Kaiseraugst, Switzerland) containing one of the following dietary lipids: (i) 7% (wt/wt) corn oil (CO); (ii) 7% borage oil providing 16.2 g of GLA/kg diet; (iii) 7% evening primrose oil (6.4 g of GLA/kg diet); (iv) 2% BO plus 5% CO (4.6 g of GLA/kg diet); (v) 5% EPO plus 2% CO (4.6 g of GLA/kg diet); (vi) 1% BO plus 6% CO (2.3 g of GLA/kg diet); (vii) 2.5% EPO plus 4.5% CO (2.3 g of GLA/kg diet). The fatty acid compositions of the dietary oils are shown in Table 1. The experimental diets were freshly prepared every two weeks and stored in tightly sealed plastic bags at −20°C. The rats were allowed free access to water and diet, and they were maintained on a 12-h light-dark cycle. Food intake was measured daily. After 6 wk, 1 mL of blood was taken by retroorbital puncture. Blood samples were allowed to clot by incubating at 37°C for 30 min, and serum was obtained to measure the production of thromboxane B_2 (TXB_2). The animals were sacrificed by withdrawing blood from the abdominal aorta under halothane anesthesia. Blood was collected into tubes containing heparin as an anticoagulant. The

TABLE 1

Fatty Acid Composition of Dietary Oils[a]

Fatty acid	CO[b]	BO[b]	EPO[b]
Saturated	15.4	16.7	10.5
Monoenoic	29.9	23.7	10.8
Polyenoic	54.8	59.6	78.7
16:0	11.5	11.9	7.6
18:0	2.6	4.0	2.0
18:1n-9	28.6	16.2	9.7
18:2n-6	53.5	36.2	69.4
18:3n-6	0.0	22.8	9.2
18:3n-3	1.1	0.3	0.3

[a] Results are expressed as the percentage of fatty acid methyl esters (mol%).

[b] CO, corn oil; BO, borage oil; EPO, evening primrose oil.

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Abbreviations: AA, arachidonic acid, 20:4n-6; BO, borage oil; CO, corn oil; DHA, docosahexaenoic acid, 22:6n-3; DHLA, dihomo-γ-linolenic acid, 20:3n-6; DPA, docosapentaenoic acid, 22:5n-3; EFA, essential fatty acid; EPO, evening primrose oil; GLA, γ-linolenic acid, 18:3n-6; HBSS, Hank's balanced salt solution; LA, linoleic acid, 18:2n-6; PGE_2, prostaglandin E_2; PGE_1, prostaglandin E_1; PUFA, polyunsaturated fatty acid(s); TXB_2, thromboxane B_2.

thoracic aorta was rapidly excised, cleared of the adhering tissues and rinsed in Hank's balanced salt solution (HBSS) to remove the blood. The aorta was then cut into rings, incubated and stimulated in 1 mL of HBSS containing 10 μM ionophore A23187 (Sigma Chemical Co., St. Louis, MO) for 5 min at 37°C, in duplicate for each animal. After centrifugation, the supernatants were removed and stored at −70°C until analyzed for prostaglandin E_2 (PGE_2) and PGE_1. The segments were blotted dry and weighed. The remaining material was stored at −70°C for fatty acid analysis. The liver was excised, quickly frozen in liquid N_2, and stored at −70°C until analyzed.

Lipid extraction and analysis. The organs were powdered in liquid nitrogen and the tissues were extracted by the method of Bligh and Dyer (39). Phospholipids were separated from total lipids by solid phase extraction with silica Bond Elut (500 mg) columns (Analytichem International, Harbor City, CA) according to the method of Hamilton and Comai (40). The fatty acids of total phospholipids were transesterified and analyzed by capillary gas chromatography on an OV-1 column as previously described (41). A standard mixture of fatty acid methyl esters was used to establish the response factors for the components identified. The fatty acids were quantified using heptadecanoic acid as internal standard. After correction for the various response factors, the amount of each fatty acid was calculated as mole percentage of the identified fatty acid peaks, which represent more than 98% of the integrated area (41).

Prostanoid analysis. Thromboxane B_2 (TXB_2) was measured using an [^{3}H]TXB_2 radioimmunoassay kit (NEK-007, New England Nuclear, Boston, MA). Serum was diluted about five hundred times with 50 mM phosphate buffer at pH 7.3, containing 0.1% gelatin. The results were expressed as ng/mL serum. PGE_2 and PGE_1 *ex vivo* synthesis was determined in the supernatants of stimulated aorta by radioimmunoassay (42). PGE_2 produced during the incubation of the thoracic aorta was analyzed using an [^{125}I]PGE_2 radioimmunoassay kit (NEK-020, New England Nuclear). The cross reactivity of the antiserum was 100% for PGE_2, 3.7% for PGE_1 and less than 0.4% for other prostaglandins. PGE_1 was measured with an antibody obtained from Pasteur Diagnostica (Marnes La-Coquette, France). Tritiated PGE_1 (NET 344, 60 Ci/mmol) was obtained from New England Nuclear, unlabeled PGE_1 from Medipro (Teufen, Switzerland). The cross-reactivity of the PGE_1 antibody was 15% against PGE_2 and less than 0.2% against other prostaglandins. The data obtained were corrected for the 15% cross reactivity of the antibody with PGE_2. The results were expressed as pg/mg aorta tissue. The measurements were in the linear portion of the standard curve.

Statistical analysis. All data are expressed as means ± SEM for animals in each diet group, with n being the number of rats. Statistical significance of mean differences between dietary groups was established by one-way analysis of variance. If significant differences were found, the Tukey's range test for multiple comparison was used to compare each group with the others. *P* values of less than 0.05 were considered significant. In order to evaluate a possible difference between the two oils, the data were analyzed by a univariate two-way analysis of variance by means of the program GLM procedure of SAS (43). For this purpose, the animals of the control group had been randomly assigned to the two treatment groups, BO and EPO. The effect of the two factors "oil" (BO resp. EPO) and "diet" (five levels of GLA) were tested for several variables. If the factor "oil" and the interaction "diet*oil" were not significant, both oils can be considered as equal for the variable analyzed when they provide the same amount of GLA.

RESULTS AND DISCUSSION

Animal characteristics. The growth of rats was similar in all dietary groups during the 6-wk feeding period. The mean food intake for the 6-wk period of the seven dietary regimes was 20.2 ± 0.2 g/d/rat. Dietary treatment had no significant effect on body weight and food consumption.

Tissue fatty acid changes. The fatty acid profiles of liver, erythrocyte and aorta phospholipids from rats fed various diets are presented in Tables 2–4. The levels of saturated fatty acids in tissue phospholipids were similar in all groups in spite of variations in the dietary saturated fatty acids. Moderate alterations were observed in the monounsaturated fatty acids. Oleic acid was slightly decreased in the groups fed EPO compared to the 7% CO diet. This was due to the lower content of 18:1n-9 in EPO (Table 1). Irrespective of any fat supplement, the total amount of polyunsaturated fatty acids (PUFA) was only modestly altered. However, the animals fed diets containing BO or EPO showed an increase in the proportion of the major PUFA of the n-6 series and a decrease in those of the n-3 series (22:5, 22:6) according to the n-3 content of the diets. In general, the fatty acid profiles of the phospholipids reflected the fatty acid content of the oils ingested by the various groups. Thus, LA levels were highest in tissues from EPO and CO fed groups and lowest in those from the BO groups reflecting the differences in the LA content of the oils (Table 1). Animals fed either BO or EPO converted GLA into DHLA, which were both incorporated in erythrocyte, liver and aorta phospholipids. The use of corn oil as dietary fat allowed us to study the effect of equal amounts of dietary GLA fed either as BO or EPO while keeping the total amount of fat constant and to compare the effect of both sources of GLA with a high supply of LA as it is usually the case in human diets, which contain up to 14 g LA/day (44). The data demonstrate that an equal amount of dietary GLA fed as BO or EPO produced a similar and selective increase in tissue DHLA levels (Tables 2–4). Furthermore, a linear dose response in the increase of DHLA and GLA was observed in liver and erythrocyte phospholipids with both BO and EPO. This is in good agreement with results obtained in human studies (30,45). Ziboh and Fletcher (45), *e.g.*, showed a dose-related increase of the DHLA content in human neutrophils of volunteers after supplementation with dietary BO. In recent years it has been debated whether the position of the fatty acids within the triacylglycerols, as found by Lawson and Hughes (46), plays a role in the absorption of GLA and other PUFA (47). In our experiment the two sources of GLA, BO or EPO, behaved identically in regard to the absorption and metabolism of GLA. The percentage of AA in erythrocyte phospholipids varied between 23.7% and 25.1% (not significant) independently of the diet, whereas in the liver a slight but significant increase from 29.9% (7% CO) to 32.6% (7% BO)

TABLE 2

Fatty Acid Composition of Liver Phospholipids[a]

Fatty acid	7% CO[b]	7% BO[b]	7% EPO[b]	2% BO 5% CO	5% EPO 2% CO	1% BO 6% CO	2.5% EPO 4.5% CO
Saturated	42.8 ± 0.5	43.5 ± 0.4	42.5 ± 0.4	42.3 ± 0.3	42.8 ± 0.4	43.5 ± 0.2	43.8 ± 0.5
16:0	19.8 ± 0.3	19.6 ± 0.3	19.9 ± 0.4	18.8 ± 0.3	20.3 ± 0.7	19.8 ± 0.4	20.0 ± 0.5
18:0	21.9 ± 0.8	23.0 ± 0.5	21.6 ± 0.7	22.7 ± 0.5	21.3 ± 0.6	22.4 ± 0.5	22.5 ± 0.7
Monoenoic	7.4 ± 0.4	7.3 ± 0.2	6.7 ± 0.2	7.4 ± 0.3	6.7 ± 0.2	7.5 ± 0.3	7.0 ± 0.3
18:1n-9	3.4 ± 0.1^{c}	2.8 ± 0.1^{d,e,f}	2.5 ± 0.1^{g}	3.2 ± 0.1^{c,e}	2.6 ± 0.1^{d,g}	3.1 ± 0.1^{c,f}	2.8 ± 0.1^{d,e,f}
18:1n-7	3.3 ± 0.3	2.9 ± 0.2	3.5 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.2
Polyenoic	49.7 ± 0.2^{c,d,e}	49.2 ± 0.3^{c,d}	50.8 ± 0.3^{e}	50.3 ± 0.3^{c,e}	50.5 ± 0.4^{e}	49.1 ± 0.1^{d}	49.2 ± 0.3^{c,d}
Total n-6	43.9 ± 0.4^{c}	46.1 ± 0.3^{d,e}	47.6 ± 0.3^{d}	45.5 ± 0.3^{e,f}	46.6 ± 0.4^{d,e}	44.1 ± 0.2^{c,f}	44.5 ± 0.5^{c,f}
18:2n-6	9.8 ± 0.3^{c}	6.3 ± 0.2^{d}	9.9 ± 0.4^{c}	9.2 ± 0.3^{c}	10.6 ± 0.2^{c}	9.4 ± 0.2^{c}	9.1 ± 0.6^{c}
18:3n-6	0.2 ± 0.0^{c}	0.8 ± 0.0^{d}	0.4 ± 0.0^{e}	0.4 ± 0.0^{c,e}	0.3 ± 0.0^{c,e}	0.3 ± 0.0^{c,e}	0.3 ± 0.0^{c,e}
20:3n-6	0.4 ± 0.0^{c}	0.9 ± 0.1^{d}	0.6 ± 0.0^{e}	0.5 ± 0.1^{e}	0.6 ± 0.0^{e}	0.5 ± 0.0^{c,e}	0.5 ± 0.0^{c,e}
20:4n-6	29.9 ± 0.2^{c,d}	32.6 ± 0.3^{e}	30.8 ± 0.3^{c,d}	31.1 ± 0.2^{c}	29.8 ± 0.4^{d}	29.9 ± 0.2^{c,d}	29.8 ± 0.3^{d}
22:4n-6	0.9 ± 0.1^{c}	1.7 ± 0.1^{d}	1.3 ± 0.1^{d,e}	1.1 ± 0.1^{c,e}	1.3 ± 0.1^{e}	1.1 ± 0.1^{c,e}	1.1 ± 0.1^{c,e}
22:5n-6	2.3 ± 0.2^{c}	3.5 ± 0.3^{c,d}	4.1 ± 0.2^{d}	2.9 ± 0.4^{c,d}	3.5 ± 0.3^{c,d}	2.5 ± 0.2^{c}	3.2 ± 0.5^{c,d}
Total n-3	5.8 ± 0.2^{c}	3.1 ± 0.0^{d}	3.2 ± 0.1^{d,e}	4.8 ± 0.1^{f}	3.9 ± 0.2^{e}	5.0 ± 0.2^{f}	4.8 ± 0.2^{f}
18:3n-3	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
22:5n-3	0.4 ± 0.0^{c,d}	0.4 ± 0.0^{c,d,e}	0.3 ± 0.0^{e}	0.4 ± 0.0^{c,d}	0.3 ± 0.0^{d,e}	0.5 ± 0.1^{c}	0.4 ± 0.0^{c,d,e}
22:6n-3	5.1 ± 0.3^{c}	2.4 ± 0.1^{d}	2.5 ± 0.1^{d,e}	3.9 ± 0.1^{f}	3.1 ± 0.1^{e}	4.2 ± 0.2^{f}	4.0 ± 0.2^{f}
20:4n-6/20:3n-6	83.4 ± 7.3^{c}	37.4 ± 1.7^{d}	50.4 ± 0.6^{d,e}	60.8 ± 4.7^{e}	53.1 ± 3.6^{d,e}	64.5 ± 2.6^{e}	61.1 ± 3.2^{e}

[a] Values expressed as mole percent of total fatty acids are means ±SEM (n = 6–8). Values within a line without a common superscript are significantly different ($P < 0.05$). Rows without any superscripts contain values that are not significantly different.

[b] CO, corn oil; BO, borage oil; EPO, evening primrose oil. The amount of fat added to the basal diet is given in weight percent of the diet.

TABLE 3

Fatty Acid Composition of Erythrocyte Phospholipids[a]

Fatty acid	7% CO[b]	7% BO[b]	7% EPO[b]	2% BO 5% CO	5% EPO 2% CO	1% BO 6% CO	2.5% EPO 4.5% CO
Saturated	48.7 ± 0.8	49.1 ± 0.7	50.4 ± 0.5	49.6 ± 0.4	49.7 ± 0.6	49.2 ± 0.5	49.0 ± 0.6
16:0	32.4 ± 0.7	32.7 ± 0.7	34.0 ± 0.8	33.0 ± 0.5	33.4 ± 0.9	32.6 ± 0.8	31.7 ± 0.6
18:0	13.6 ± 0.2	14.0 ± 0.2	13.6 ± 0.5	13.9 ± 0.3	13.6 ± 0.3	13.9 ± 0.5	14.5 ± 0.2
Monoenoic	11.4 ± 0.2^{c}	11.6 ± 0.2^{c}	9.4 ± 0.2^{d}	11.4 ± 0.2^{c}	9.8 ± 0.3^{d,e}	11.5 ± 0.2^{c}	10.7 ± 0.3^{c,e}
18:1n-9	6.6 ± 0.1^{c,d}	5.9 ± 0.1^{e}	4.8 ± 0.2^{f}	6.4 ± 0.1^{c,e}	5.3 ± 0.1^{f}	6.6 ± 0.2^{c}	6.0 ± 0.1^{d,e}
18:1n-7	3.1 ± 0.1	2.9 ± 0.1	3.3 ± 0.1	3.0 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1
Polyenoic	39.9 ± 0.7	39.4 ± 0.5	40.2 ± 0.6	39.0 ± 0.4	40.5 ± 0.6	39.3 ± 0.4	40.3 ± 0.5
Total n-6	37.6 ± 0.7	38.0 ± 0.5	38.7 ± 0.6	37.1 ± 0.4	38.8 ± 0.6	37.1 ± 0.4	38.3 ± 0.5
18:2n-6	8.2 ± 0.2^{c,d}	5.7 ± 0.2^{e}	8.4 ± 0.2^{c}	7.5 ± 0.2^{d}	8.6 ± 0.1^{c}	7.8 ± 0.2^{c,d}	7.9 ± 0.2^{c,d}
18:3n-6	0.1 ± 0.0^{c}	0.6 ± 0.0^{d}	0.2 ± 0.0^{e}	0.2 ± 0.0^{e,f}	0.2 ± 0.0^{f}	0.1 ± 0.0^{g}	0.1 ± 0.0^{g}
20:3n-6	0.4 ± 0.0^{c}	0.8 ± 0.1^{d}	0.6 ± 0.0^{e}	0.5 ± 0.0^{c,e}	0.5 ± 0.0^{c,e}	0.4 ± 0.0^{c}	0.4 ± 0.0^{c,e}
20:4n-6	24.1 ± 0.4	25.1 ± 0.3	23.7 ± 0.5	24.1 ± 0.3	23.8 ± 0.6	23.8 ± 0.3	24.8 ± 0.4
22:4n-6	2.9 ± 0.2^{c}	3.8 ± 0.1^{d}	3.4 ± 0.1^{c,d}	2.9 ± 0.1^{c}	3.4 ± 0.2^{c,d}	3.0 ± 0.1^{c}	2.9 ± 0.1^{c}
22:5n-6	1.4 ± 0.1^{c}	1.7 ± 0.1^{c,d}	2.0 ± 0.1^{d}	1.6 ± 0.1^{c,d}	1.9 ± 0.1^{c,d}	1.5 ± 0.1^{c,d}	1.7 ± 0.2^{c,d}
Total n-3	2.4 ± 0.0^{c}	1.4 ± 0.0^{d}	1.5 ± 0.1^{d,e}	1.9 ± 0.0^{f,g}	1.8 ± 0.0^{f,e}	2.2 ± 0.1^{c,g}	2.0 ± 0.1^{f,g}
18:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:5n-3	0.6 ± 0.0^{c,d}	0.4 ± 0.0^{e,f}	0.4 ± 0.0^{f}	0.5 ± 0.0^{d,e}	0.4 ± 0.0^{e,f}	0.6 ± 0.0^{c}	0.5 ± 0.0^{c,d}
22:6n-3	1.6 ± 0.0^{c}	0.8 ± 0.0^{d}	0.9 ± 0.1^{d,e}	1.2 ± 0.0^{f,g}	1.1 ± 0.0^{e,g}	1.3 ± 0.1^{f}	1.3 ± 0.1^{f,g}
20:4n-6/20:3n-6	65.3 ± 3.9^{c}	33.1 ± 2.1^{d}	43.2 ± 1.3^{e}	48.9 ± 1.8^{e,f}	50.0 ± 1.6^{e,f,g}	56.9 ± 1.0^{c,f,g}	58.8 ± 2.6^{c,g}

[a] Values expressed as mole percent of total fatty acids are means ±SEM (n = 6–8). Values within a line without a common superscript are significantly different ($P < 0.05$). Rows without any superscripts contain values that are not significantly different.

[b] CO, corn oil; BO, borage oil; EPO, evening primrose oil. The amount of fat added to the basal diet is given in weight percent of the diet.

was observed (Table 2). EPO had no effect on the AA level. In the aorta, there was a similar increase from 24.4% (7% CO) to 26.3% (7% BO), but in contrast to the liver there was no dose relationship between the AA level and BO content in the diet. Although the DHLA levels are only about 1 to 6% of AA levels, the increase in DHLA in the three tissues tested followed the GLA content in the diet. The highest values were always obtained with 7% BO. The AA/DHLA ratio of phospholipids was reduced with both BO and EPO diets independently of the source of GLA (Tables 2–4). Due to the low n-3 fatty acid content of those diets, EPA was almost not detectable in the tissues analyzed. When rats were fed diets containing EPO or BO, the proportion of 22:6n-3 was decreased and that of 22:5n-6 and 22:4n-6 increased as reported previously (25). Those changes were mainly due to a shift in the dietary n-3/n-6 ratio and thus confirmed the well known competition between n-6 and n-3 fatty acids for desaturases and

TABLE 4

Fatty Acid Composition of Aorta Phospholipids[a]

Fatty acid	7% CO[b]	7% BO[b]	7% EPO[b]	2% BO 5% CO	5% EPO 2% CO	1% BO 6% CO	2.5% EPO 4.5% CO
Saturated	48.6 ± 0.3^{c,d}	48.5 ± 0.2^{c,d}	48.9 ± 0.1^{d}	48.3 ± 0.4^{c,d}	47.8 ± 0.2^{c,d}	47.4 ± 0.2^{c}	49.1 ± 0.4^{d}
16:0	24.9 ± 0.2^{c,d}	25.0 ± 0.3^{c,d}	25.2 ± 0.2^{c,d}	24.7 ± 0.2^{d}	24.4 ± 0.1^{d}	24.3 ± 0.3^{d}	26.1 ± 0.2^{c}
18:0	20.0 ± 0.3	20.1 ± 0.3	20.4 ± 0.2	20.6 ± 0.2	20.4 ± 0.1	20.1 ± 0.3	19.8 ± 0.2
Monoenoic	12.8 ± 0.2^{c}	11.7 ± 0.2^{d}	10.8 ± 0.3^{d}	11.7 ± 0.3^{c,d}	10.7 ± 0.4^{d}	11.7 ± 0.2^{d}	11.5 ± 0.1^{d}
18:1n-9	6.6 ± 0.1^{c}	5.7 ± 0.1^{d,e,f}	4.9 ± 0.1^{g}	5.7 ± 0.1^{e}	5.1 ± 0.2^{f,g}	5.9 ± 0.1^{d,e}	5.5 ± 0.1^{d,e,f,g}
18:1n-7	4.3 ± 0.1	4.0 ± 0.0	4.3 ± 0.1	4.3 ± 0.2	4.1 ± 0.1	4.1 ± 0.1	4.3 ± 0.1
Polyenoic	38.6 ± 0.4^{c}	39.8 ± 0.3^{c,d}	40.3 ± 0.4^{d,e}	39.9 ± 0.2^{c,d}	41.5 ± 0.4^{e}	40.9 ± 0.3^{d,e}	39.5 ± 0.4^{c,d}
Total n-6	36.2 ± 0.4^{c}	38.0 ± 0.3^{d,e}	38.6 ± 0.4^{d,e}	37.8 ± 0.2^{d}	39.6 ± 0.4^{e}	38.6 ± 0.4^{d,e}	37.3 ± 0.4^{c,d}
18:2n-6	3.6 ± 0.1^{c}	2.2 ± 0.2^{d}	3.8 ± 0.2^{c}	3.2 ± 0.2^{c}	3.7 ± 0.1^{c}	3.4 ± 0.2^{c}	3.5 ± 0.1^{c}
18:3n-6	0.1 ± 0.0^{c}	0.1 ± 0.0^{d}	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}
20:3n-6	0.9 ± 0.0^{c}	1.5 ± 0.1^{d}	1.3 ± 0.1^{e}	1.1 ± 0.0^{e,f}	1.2 ± 0.0^{e,f}	1.1 ± 0.0^{c,e}	1.0 ± 0.0^{c,e}
20:4n-6	24.4 ± 0.3^{c}	26.3 ± 0.3^{d}	25.6 ± 0.4^{c,d}	25.7 ± 0.2^{c,d}	26.4 ± 0.4^{d}	26.3 ± 0.4^{d}	25.3 ± 0.4^{c,d}
22:4n-6	4.6 ± 0.1^{c}	5.3 ± 0.2^{d}	5.0 ± 0.1^{c,d}	5.1 ± 0.1^{c,d}	5.2 ± 0.1^{c,d}	5.0 ± 0.1^{c,d}	4.7 ± 0.1^{c,d}
22:5n-6	1.8 ± 0.1^{c}	2.2 ± 0.2^{c,d}	2.2 ± 0.1^{c,d}	2.0 ± 0.1^{c,d}	2.3 ± 0.1^{d}	2.1 ± 0.1^{c,d}	2.0 ± 0.1^{c,d}
Total n-3	2.4 ± 0.0^{c}	1.8 ± 0.1^{d}	1.7 ± 0.0^{d}	2.2 ± 0.0^{c,e}	2.0 ± 0.1^{d,e}	2.3 ± 0.1^{c}	2.2 ± 0.1^{c,e}
18:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
22:5n-3	0.4 ± 0.0^{c}	0.4 ± 0.0^{c,d,e}	0.3 ± 0.0^{d}	0.4 ± 0.0^{c}	0.3 ± 0.0^{d,e}	0.5 ± 0.0^{c}	0.4 ± 0.0^{c,e}
22:6n-3	1.8 ± 0.0^{c}	1.3 ± 0.1^{d}	1.3 ± 0.0^{d}	1.6 ± 0.0^{c,e}	1.5 ± 0.1^{d,e}	1.7 ± 0.1^{c}	1.6 ± 0.1^{c,e}
20:4n-6/20:3n-6	27.4 ± 0.9^{c}	17.7 ± 0.7^{d}	20.2 ± 1.0^{d,e}	23.0 ± 0.9^{e,f}	22.8 ± 0.7^{e,f}	25.1 ± 1.0^{c,f}	25.7 ± 1.1^{c,f}

[a]Values expressed as mole percent of total fatty acids are means ±SEM (n = 6–8). Values within a line without a common superscript are significantly different ($P < 0.05$). Rows without any superscripts contain values that are not significantly different.

[b]CO, corn oil; BO, borage oil; EPO, evening primrose oil. The amount of fat added to the basal diet is given in weight percent of the diet.

acyltransferases (48). The interactions between n-3 PUFA and GLA-containing oils have been reported previously (31–35), showing that a combination of fish oil and GLA-containing oil is needed to achieve the highest 20:3n-6/AA ratios in tissue phospholipids.

An overall effect of the factors "oil" (EPO and BO) and "diet" (five levels of GLA) on the n-3 or n-6 fatty acids was tested by means of multivariate analysis of variance where the vectors of the dependent variables were either 18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6 for the n-6 series or 18:3n-3, 22:5n-3 and 22:6n-3 for the n-3 series. The factor "oil" had no significant effect for the n-6 fatty acids in all tissue tested (liver $P = 0.078$, aorta $P = 0.374$, erythrocytes $P = 0.053$), whereas for the n-3 fatty acids the differences were significant in liver and aorta (liver $P = 0.007$, aorta $P = 0.008$, erythrocytes $P = 0.252$). The factor "diet" was significant in all cases. The interactions between the two factors and particular fatty acids have been calculated by two-way analysis of variance (Table 5). In liver, the factor "oil" had a significant effect on total n-3 fatty acids which can be explained by the difference in DHA levels. The factor "diet" was significant for all fatty acids. An interaction "diet*oil" was observed regarding AA and docosapentaenoic acid (DPA). In aorta, a similar pattern was found as in the liver. The n-3 fatty acids depend on the factor "oil" due to DPA and DHA, and all fatty acids except 22:5n-6 were dependent on the factor "diet." An interaction "diet*oil" was also seen regarding AA. The erythrocytes behaved differently: the factor "oil" had a significant effect on the n-6 fatty acids due to the 22:4n-6 but not on the n-3 series. The factor "diet" had a significant effect on all fatty acids except on total n-6 fatty acids, 20:4n-6, 22:5n-6 and on the ratio AA/DHLA. Here, an interaction "diet*oil" was seen with regard to 18:2n-6 and 22:4n-6. In all cases, LA, GLA and DHLA were only dependent on the factor "diet" but not on the factor "oil," and there were no interactions with the exception of LA in erythrocytes. This means that the contents of GLA and DHLA in phospholipids depend only on the GLA content in the diet and not on the type of oil supplemented. An interference with dietary LA, as it could be expected from the different LA content in BO and EPO, has been avoided by feeding a diet already rich in LA. However, the total amount of n-3 fatty acids in the diet was very low, therefore the significant differences of n-3 fatty acids in the phospholipids can be explained by the slightly higher content of 18:3n-3 in BO.

Prostanoid levels. PGE_2 levels in stimulated aorta and TXB_2 levels in serum were not significantly influenced by dietary GLA-rich oils. PGE_1 levels were increased in stimulated aorta in all the groups fed GLA compared to the corn oil diet (Table 6). Two-way analysis of variance indicated a significant increase of PGE_1 by dietary GLA ($P = 0.006$), whereas neither the factor "oil" nor the interaction between the two factors had a significant influence on PGE_1 levels (Table 6). Previously, an increased production of PGE_1 was observed in epidermis, aorta and platelets after supplementing with GLA (23,49–52). Moreover, Ziboh and co-workers (22–24) observed a significant increase in 15-hydroxyeicosatrienoic acid content in the epidermis of BO fed animals. 15-Hydroxyeicosatrienoic acid is a potent inhibitor of 5- and 12-lipoxygenases (24). Due to the beneficial effects attributed to PGE_1, an increased production may be of relevance in atherosclerosis and inflammation (2,16–24). Recently, Ziboh and Fletcher (45) showed that the conversion of AA to leukotriene B_4, a potent pro-inflammatory metabolite, was inhibited by dietary BO in human polymorphonuclear neutrophils of healthy volunteers.

TABLE 5

P Values of the Univariate Two-Way Analysis of Variance

Factors	Oil[a]	Diet	Diet*Oil
Liver			
Total n-6	0.137	0.0001	0.463
18:2n-6	0.154	0.0001	0.062
18:3n-6	0.710	0.0001	0.540
20:3n-6	0.333	0.0001	0.920
20:4n-6	0.342	0.0001	0.032
20:4n-6/20:3n-6	0.400	0.0001	0.620
22:4n-6	0.918	0.0001	0.166
22:5n-6	0.564	0.012	0.228
Total n-3	0.009	0.0001	0.109
22:5n-3	0.261	0.0021	0.021
22:6n-3	0.017	0.0001	0.223
Erythrocyte			
Total n-6	0.012	0.649	0.897
18:2n-6	0.083	0.0001	0.010
18:3n-6	0.497	0.0001	0.927
20:3n-6	0.341	0.0001	0.350
20:4n-6	0.181	0.072	0.207
20:4n-6/20:3n-6	0.557	0.908	0.843
22:4n-6	0.020	0.0001	0.025
22:5n-6	0.216	0.080	0.499
Total n-3	0.741	0.0001	0.141
22:5n-3	0.239	0.0001	0.707
22:6n-3	0.555	0.0001	0.093
Aorta			
Total n-6	0.955	0.0001	0.0002
18:2n-6	0.199	0.0001	0.399
18:3n-6	0.601	0.0004	0.627
20:3n-6	0.996	0.0001	0.488
20:4n-6	0.463	0.010	0.049
20:4n-6/20:3n-6	0.754	0.0001	0.740
22:4n-6	0.547	0.017	0.380
22:5n-6	0.434	0.149	0.199
Total n-3	0.042	0.0001	0.427
22:5n-3	0.027	0.0001	0.942
22:6n-3	0.030	0.0023	0.180

[a]Two-way analysis of variance for the following factors. Oil; two levels: borage and evening primrose oil. Diet; five levels: 0.0, 2.3, 4.6, 6.4 and 16.2 g of GLA/kg diet. Diet*Oil; interactions between diet and oil.

Results of this and other studies, therefore, show that the levels of GLA and DHLA in tissue phospholipids can be significantly increased by feeding either BO or EPO, resulting in lower AA/DHLA ratios in phospholipids (22,24,33,35,37,45). Moreover, when equal amounts of GLA were fed, either as BO or as EPO (group 4, 5 and 6, 7), no significant differences were observed in the tissue GLA or DHLA levels. The data suggest a dose-related effect between the amount of dietary GLA ingested and the levels of GLA, DHLA and AA/DHLA ratios measured in the tissues. Finally, the selective increase of the levels of DHLA, with either BO or EPO, produced a small increase in the *ex vivo* production of PGE_1 in the aorta.

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TABLE 6

Effect of Dietary Oils on Prostanoid Production[a]

	7% CO[b]	7% BO[b]	7% EPO[b]	2% BO + 5% CO	5% EPO + 2% CO	1% BO + 6% CO	2.5% EPO + 4.5% CO
Aorta							
PGE_2[c] (pg/mg aorta)	126.7 ± 11.6	125.9 ± 10.6	141.2 ± 11.1	132.4 ± 8.1	123.5 ± 15.2	128.3 ± 10.1	114.5 ± 7.2
PGE_1[c] (pg/mg aorta)	10.5 ± 0.3	14.0 ± 0.6	14.9 ± 0.6	13.7 ± 1.0	12.8 ± 1.3	13.4 ± 0.7	13.5 ± 0.7
Serum							
TXB_2[c] (ng/mL)	269.7 ± 12.8	351.7 ± 45.4	329.2 ± 23.5	347.7 ± 41.3	297.3 ± 37.0	340.5 ± 37.0	339.5 ± 26.7

[a]Values are expressed as means ±SEM (n = 6-8).

[b]CO, corn oil; BO, borage oil; EPO, evening primrose oil. The amount of fat added to the basal diet is given in weight percent of the diet.

[c]Two-way analysis of variance showed no effect of dietary γ-linolenic acid (GLA) on PGE_2 and TXB_2 formation. However, PGE_1 synthesis was significantly increased by dietary GLA ($P = 0.006$) but no difference between the oils was observed ($P = 0.98$).

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Nonessential Fatty Acids in Formula Fat Blends Influence Essential Fatty Acid Metabolism and Composition in Plasma and Organ Lipid Classes in Piglets

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The n-6 and n-3 fatty acid status of developing organs is the cumulative result of the diet lipid composition and many complex events of lipid metabolism. Little information is available, however, on the potential effects of the saturated fatty acid chain length (8:0–16:0) or oleic acid (18:1) content of the diet on the subsequent metabolism of the essential fatty acids 18:2n-6 and 18:3n-3 and their elongated/desaturated products. The effects of feeding piglets formulas with fat blends containing either coconut oil (12:0 + 14:0) or medium chain triglycerides (MCT, 8:0 + 10:0) but similar levels of 18:1, 18:2n-6 and 18:3n-3, or MCT with high or low 18:1 but constant 18:2n-6 and 18:3n-3 on the fatty acid composition of plasma, liver and kidney triglycerides, phospholipids and cholesteryl esters, and of brain total lipid, were studied. Diet-induced changes in the fatty acid composition of lipid classes were generally similar for plasma, liver and kidney. Dietary 18:1 content was reflected in tissue lipids and was inversely associated with levels of 18:2n-6. Lower percentage of 18:2n-6, however, was not associated with lower levels of its elongated/desaturated product 20:4n-6 but was associated with higher levels of 22:6n-3. Feeding coconut oil *vs.* MCT resulted in lower 18:1 levels in all lipids, and higher percentages of 20:4n-6 in tissue phospholipid. Increasing the dietary n-6/n-3 ratio from 5 to 8 significantly increased tissue percentage of 18:2n-6 and decreased phospholipid 22:6n-3. In contrast to plasma, liver and kidney, brain lipid fatty acid composition was not influenced by the formula saturated fatty acid chain length, content of 18:1, or n-6/n-3 ratio. In summary, the studies show that the dietary requirement for n-6 and n-3 fatty acids may be influenced by the nonessential saturated and monounsaturated fatty acids fed concurrently.
Lipids 27, 1024–1031 (1992).

Formulas presently available for infant nutrition commonly use blends of vegetable oils, sometimes with the addition of oleo oils, as their source of fat. Although almost all formulas provide the essential fatty acids linoleic (18:2n-6) and linolenic (18:3n-3), levels of oleic acid (18:1) and the chain length of the saturated fatty acids may differ widely. Infant formulas often use coconut or medium chain triglyceride (MCT) oils as a preferred source of saturated fat, usually for term and preterm formulas, respectively. The medium chain fatty acids (MCFA) in MCT oils are more soluble in water than long chain fatty acids (LCFA, C > 14), thus facilitating fat absorption when intraluminal hydrolysis or micelle formation is compromised (1,2). The absorption of triglycerides containing LCFA, by contrast, requires intraluminal hydrolysis of the triglycerides to free fatty acids and monoglycerides, and micelle formation for absorption (1). Monoglycerides and LCFA are reassembled into triglycerides in the enterocyte and secreted into the lymph as components of chylomicrons, thus initially by-passing the liver. In contrast, MCFA are predominantly transported directly to the liver *via* the portal vein (1,2). In the liver, MCFA cross the mitochondrial membrane independent of carnitine (3), and provide a readily available source of fatty acids for oxidation (4,5). In addition, since MCFA are not good substrates for fatty-acid binding protein (6), they are not readily activated to fatty acyl-CoA for incorporation into hepatic lipid pools (4,5,7). It is not known whether these differences in metabolic handling of dietary MCFA and LCFA influence the fate of the accompanying dietary essential fatty acids, specifically their shunting either to oxidation or to acylation into triglyceride (TG), phospholipid (PL) or cholesteryl ester (CE).

Desaturation of 18:1n-9, 18:2n-6 and 18:3n-3 is subject to competitive inhibition, with preferential desaturation occurring in the relative order of n-3 > n-6 > n-9 fatty acids (8). Human milk fat typically contains about 3% (w/w of total fatty acids) 16:1 and about 30–40% 18:1, whereas infant formula fat blends vary widely with 10–40% 18:1 and no appreciable 16:1. Human milk fat also contains small amounts (<1%) of the long chain polyunsaturated fatty acids (LCP) 20:4n-6 and 22:6n-3. The importance of these n-6 and n-3 LCP for normal tissue development and function is being increasingly recognized (9,10). It is well documented that formula feeding, compared with human milk feeding, results in lower plasma and red blood cell (RBC) PL levels of the elongated/desaturated derivatives of 18:2n-6 and 18:3n-3, namely 20:4n-6 and 22:6n-3 (11–14). The possible effect of the monounsaturated fatty acid content of the formula on n-6 and n-3 fatty acid metabolism, however, has received little attention.

The two-fold objective of the present study was to examine the effect on specific tissues of diets containing (i) MCT or coconut oil with 8:0 + 10:0 (MCFA) or 12:0 + 14:0 (intermediate chain fatty acids, ICFA), respectively, as the predominant source of saturated fatty acids, and with similar levels of 18:1, 18:2n-6 and 18:3n-3 and (ii) MCT oil with low and high levels of 18:1, and similar quantities of 18:2n-6 and 18:3n-3. The fatty acid composition of piglet plasma, liver and kidney PL, TG and CE, and brain total lipid were analyzed.

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Abbreviations: ANOVA, analysis of variance; CE, cholesteryl ester; EDTA, tetrasodium ethylenediaminetetraacetic acid; FA, fatty acid; ICFA, intermediate chain fatty acids; LCFA, long chain fatty acids (C > 14); LCP, long chain polyunsaturated fatty acids (C_{20} and C_{22} with ≥2 double bonds); MCFA, medium chain fatty acids; MCT, medium chain triglyceride; PL, phospholipid; RBC, red blood cell; TG, triglyceride; TRIS, *tris*(hydroxymethyl)methylamine; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Animals and diets. Male Yorkshire piglets, delivered at 116–118 d of gestation, were obtained from Jansen Farm (Abbotsford, British Columbia, Canada) and Peter Hill Holdings (Langley, British Columbia, Canada). Six piglets, each born to a different sow, were kept at the farm and suckled by their natural mothers for 18 d. These piglets provided a reference group for formula fed animals. An additional 24 piglets were taken from their mothers immediately after birth, and were housed in the animal care quarters at the University of British Columbia (Shaughnessy Site). Six piglets were randomly assigned to each of four dietary groups, and no group had more than one piglet born to the same sow. The piglets were bottle-fed every 3–4 h for 18 d from birth, and porcine serum immunoglobulins were added to the liquid diets for the first 72 h after birth to provide passive immunity (15).

The liquid piglet diets were manufactured by Mead Johnson (Evansville, IN), and were based on commercial infant formula modified to meet the nutrient requirements of growing piglets (16). The formula fat blends are given in Table 1 and are designated diets A, B, C and D. The comparisons discussed in this paper will focus on the percentage of dietary 18:1 and the chain length of the predominant saturated fatty acid. Formulas A and B contained 37% MCFA + 11% 18:1 and 18% MCFA + 36% 18:1, respectively, with similar amounts of 18:2n-6 and 18:3n-3. Formulas C and D compared MCT *vs.* coconut oil in the fat blend, and contained similar amounts of 18:1, 18:2n-6 and 18:3n-3.

The studies were approved by the Animal Care Committee at the University of British Columbia, and conformed with Canadian Council on Animal Care guidelines for the care and use of animals for research.

Sample collection and analysis. After an overnight fast of 10–12 h, the piglets were anaesthetized with ketamine/rompun (37.5/3.75 mg/kg, respectively) by intramuscular injection. Blood was withdrawn by cardiac puncture into ethylenediaminetetraacetic acid (EDTA)-rinsed syringes and the animals sacrificed by intracardiac injection of 10 mL 1M KCl. Plasma was separated by centrifugation and the liver, kidney and brain were rapidly excised and weighed. The entire liver and brain were quickly homogenized in ice-cold sucrose buffer [225 mM sucrose, 25 mM tris(hydroxymethyl)methylamine (TRIS), 10 mM glutathione, 500 mg/mL leupeptin and 2 μg/mL aprotinen]. Kidneys were prepared by removing the capsule and fibrous cortex, and the organs were then frozen in liquid nitrogen and stored at −80°C until further processing.

Kidneys were homogenized in saline, and aliquots of liver, kidney and brain homogenate were removed for determination of protein concentration (17) and for lipid analysis. Plasma and organ lipids were extracted according to Folch *et al.* (18), aliquots dried under nitrogen, reconstituted in isopropyl alcohol and total cholesterol, triglycerides (Diagnostic Chemicals Inc., North Vancouver, British Columbia, Canada) and free cholesterol (Boehringer Mannheim, Laval, Quebec, Canada) determined with enzymatic reagents. Phospholipid, triglyceride and cholesteryl esters from plasma, liver and kidney lipid extracts were separated by thin-layer chromatography on Silica Gel 60 plates (0.5 mm; E. Merck, Darmstadt, Germany) using petroleum hydrocarbon (b.p. 30–60°C)/diethyl ether/glacial acetic acid (85:15:3, vol/vol/vol) as the solvent system. Lipid classes were recovered from the silica gel by extraction with chloroform/methanol (1:1, vol/vol), heptadecanoic acid (Sigma Chemical Co., St. Louis, MO) was added as internal standard for fatty acid analysis, and the samples were dried under nitrogen. Phospholipid fatty acid methyl esters were prepared in 1 mL methanol/conc. HCl (5:1, vol/vol) at 100°C for 5 min, and triglyceride and cholesteryl ester fatty acid methyl esters in 1 mL of 14% boron trifluoride in methanol, at 100°C for 45 min. Fatty acid methyl esters were recovered in pentane, dried under nitrogen, and resuspended in hexane for separation by gas-liquid chromatography (Varian 3400 Gas Chromatograph, Georgetown, Ontario, Canada, with an Rtx 2330 capillary column, 30 m × 0.25 mm i.d., Restek Corporation, Bellefonte, PA) and quantification using a Varian Star Integrator data system. Identification of fatty acids was based on the retention times of authentic standards (Sigma Chemical Company; Supelco Inc., Bellefonte, PA; Nu-Chek-Prep Inc., Elysian, MN). The fatty acid composition of the piglet diets was analyzed similarly, following preparation of methyl esters by the method of Lepage and Roy (19).

Statistical analysis. Comparisons were made between the formula diets A, B, C and D and between formula and sow-fed controls using analysis of variance (ANOVA) and Fisher's least square difference tests (Number Cruncher Statistical System, Version 5.1, Kaysville, UT). Differences were considered significant at the equivalent of $P < 0.05$, after correction with the Bonferroni factor for the number of comparisons made. Values given are means ± SE for the number of animals indicated.

TABLE 1

Composition of Dietary Fat Blends[a]

	Diet			
	A	B	C	D
Oils (% w/w of total)				
Medium chain triglyceride	40	20	31	
Soy	40	40		
Coconut	20	10		34.7
High-oleic safflower		30	26.1	23.1
Canola			33.3	33.3
Safflower			9.6	8.8
Fatty acids (% w/w of total)				
8:0 + 10:0	37	18	30	5
20:0 + 14:0	13	6	0.7	26
16:0 + 18:0	11	12	7	10
18:1	11	36	41	39
18:2n-6	24.0	25.6	16.2	15.6
18:3n-3	2.9	3.2	3.4	3.2
n-6/n-3 ratio	8.3	8.0	4.8	4.9

[a]The macronutrient content of the formulas was similar to that of sow milk and provided 58 g/L protein, 67 g/L fat and 60 g/L carbohydrate with a total caloric density of 1075 kcal/L.

RESULTS

Body weights were not significantly different among the groups of 18- d-old piglets fed the formulas (mean value for all formula groups, 4136 + 314 g, n = 24) but were

significantly lower than sow-fed piglets (5085 ± 176, n = 6; $P < 0.05$). Liver and kidney weights were not significantly different between any of the groups.

Quantitation of plasma TG, total cholesterol and free cholesterol revealed no significant differences among the groups of formula-fed piglets; total cholesterol and free cholesterol levels, however, were significantly lower in the formula-fed than in the sow-fed animals (mean value for all formula groups, 79 ± 5 and 23 ± 2 mg/dL respectively, and for sow-fed, 174 ± 7 and 50 ± 5 mg/dL, respectively). No significant differences in the concentration of TG, PL, total cholesterol or free cholesterol (mg/g protein) were found in the liver, kidney or brain among any of the formula-fed groups or between formula-fed and sow-fed animals.

The design of the experiments allows for comparisons between formulas with similar levels of 18:2n-6 and 18:3n-3 but in which the percentage of 18:1 was increased threefold at the expense of MCFA (group A *vs.* B) and between formulas with similar 18:1, 18:2n-6 and 18:3n-3 but with MCFA or ICFA as the predominant source of saturated fatty acids (group C *vs.* D). The effect of differences in n-6/n-3 ratio (reflecting the ratio of 18:2n-6 to 18:3n-3, which are known to compete with each other for desaturation and acylation) can also be addressed by comparing diets A and B, in which the n-6/n-3 ratio was approximately 8, with diets C and D which had n-6/n-3 ratios of approximatey 5 (Table 1). Analyses from piglets fed sow milk were done concurrently and are included for reference. The sow milk contained (% w/w of total fatty acids) 0.18% 8:0 + 10:0, 3.80% 12:0 + 14:0, 34.90% 16:0 + 18:0, 37.54% 18:1, 11.08% 18:2n-6, 1.10% 18:3n-3, 1.05% LCPn-6 and 0.44% LCPn-3, with an n-6/n-3 ratio of 7.9.

Comparisons between formula-fed and sow-fed piglets. Results of analyses of piglets suckled by their natural mothers, or fed formula are included in Tables 2–5 and Figures 1–3. Comparisons between formula-fed and sow-fed piglets are considered in brief, with subsequent more detailed discussion given to differences between the formula groups.

Several consistent differences in plasma and tissue lipid composition are evident between sow-fed and formula-fed piglets. The plasma TG, PL and CE percentage of 16:0 was lower and percentage of 18:2n-6 higher in all the formula than sow-fed piglets (Fig. 1). Plasma PL percentage of 20:4n-6 was also lower in all the formula groups compared to the sow-fed group. In addition, piglets fed diets with n-6/n-3 ratios of about 8 (A and B), had lower plasma PL percentage of 22:6n-3 than sow-fed controls, but piglets fed diets with n-6/n-3 ratios of about 5 (C and D) had similar plasma PL percentage of 22:6n-3 to the sow-fed group (Fig. 1). Differences in the fatty acid composition of plasma and kidney lipids were similar between formula-fed and sow-fed animals, but patterns were not always similar in the liver. Thus, in contrast to plasma, the liver CE percentage of 16:0 of formula-fed piglets was not different from the sow-fed group, neither was the liver CE percentage of 18:2n-6 altered by feeding diets C or D. Also unlike the plasma, the liver PL levels of 18:2n-6 in piglets fed diet D, 20:4n-6 in piglets fed diets A or D, and 22:6n-3 in all the formula groups were not different from the sow-fed group. Levels of 18:3n-3 were consistently higher in all lipid fractions of the plasma, liver and kidney for formula *vs.* sow-fed animals, and for a given diet treatment the percentage of 18:3n-3 was higher in the PL and CE than TG fraction. This can be seen from the difference in

TABLE 2

Effect of Dietary Fat Composition on Fatty Acid Composition of Plasma Lipid Classes[a]

Diet	Σ Saturated FA	Σ Monounsaturated FA	Σ n-6	Σ n-3	Σ LCPn-6	Σ LCPn-3
			(% wt of total fatty acids)			
Triglycerides						
Sow-fed	34.4 ± 0.7	47.9 ± 0.7	15.2 ± 0.7	2.5 ± 0.1	2.5 ± 0.4	1.1 ± 0.1
A	32.4 ± 1.4^{a}	21.3 ± 0.8^{a,x}	41.0 ± 1.5^{a,x}	5.3 ± 0.6^{a,x}	3.5 ± 0.8^{a}	1.4 ± 0.4^{a}
B	28.3 ± 1.9^{a,b,x}	41.0 ± 1.8^{b,x}	28.2 ± 0.7^{b,x}	2.4 ± 0.3^{b}	1.9 ± 0.4^{a,b}	0.4 ± 0.1^{b,c}
C	16.1 ± 0.6^{c,x}	53.8 ± 0.7^{c,x}	25.2 ± 0.5^{b,c,x}	4.9 ± 0.2^{a,x}	1.4 ± 0.2^{b}	1.0 ± 0.1^{a,c}
D	25.9 ± 0.7^{b,x}	46.0 ± 1.2^{d}	23.9 ± 0.8^{c,x}	4.2 ± 0.4^{a}	1.7 ± 0.1^{a,b}	1.0 ± 0.1^{a,c}
Phospholipids						
Sow-fed	45.5 ± 0.5	15.7 ± 0.5	31.8 ± 0.8	7.0 ± 0.1	18.6 ± 0.6	6.7 ± 0.2
A	47.8 ± 1.0^{a}	8.3 ± 0.6^{a,x}	39.5 ± 0.4^{a,x}	4.4 ± 0.4^{a,x}	8.9 ± 0.6^{a,x}	3.6 ± 0.4^{a,x}
B	43.1 ± 0.4^{b}	17.0 ± 0.5^{b}	35.5 ± 0.3^{b,x}	4.4 ± 0.2^{a,x}	9.1 ± 0.6^{a,b,x}	3.5 ± 0.3^{a,x}
C	39.9 ± 0.6^{c,x}	22.4 ± 0.7^{c,x}	31.8 ± 0.4^{c}	6.0 ± 0.3^{b}	9.4 ± 0.6^{a,b,x}	4.9 ± 0.4^{a,b,x}
D	42.9 ± 0.4^{b}	18.3 ± 0.6^{b}	32.7 ± 0.1^{c}	6.1 ± 0.3^{b}	11.7 ± 0.9^{b,x}	5.2 ± 0.4^{b}
Cholesteryl esters						
Sow-fed	24.1 ± 0.3	33.0 ± 0.6	41.7 ± 0.7	0.9 ± 0.0	5.2 ± 0.2	0.4 ± 0.0
A	13.9 ± 0.6^{a,b,x}	11.0 ± 0.2^{a,x}	72.6 ± 0.8^{a,x}	2.3 ± 0.1^{b,x}	3.9 ± 0.4^{x}	0.5 ± 0.1^{a,b}
B	14.5 ± 0.4^{b,x}	22.8 ± 0.2^{b,x}	60.7 ± 0.6^{b,x}	1.8 ± 0.1^{a,x}	3.4 ± 0.3^{x}	0.3 ± 0.0^{a}
C	11.8 ± 0.2^{a,x}	30.8 ± 1.1^{c}	54.4 ± 1.1^{c,x}	2.7 ± 0.0^{c,x}	3.3 ± 0.2^{x}	0.6 ± 0.1^{b,x}
D	14.5 ± 0.8^{b,x}	27.6 ± 0.6^{d,x}	55.1 ± 0.5^{c,x}	2.5 ± 0.1^{b,c,x}	3.7 ± 0.2^{x}	0.4 ± 0.0^{a,b}

[a]Values are means ± SE for four (sow-fed) or six (formula) animals per group. Values within groups A, B, C and D without a common superscript (a, b, c or d) are significantly different from other formula groups; x, significantly different from the sow-fed group ($P < 0.05$, corrected with Bonferroni factor). Σ Saturated fatty acid (FA), total saturates (including 12:0, 14:0, 16:0, 18:0, 20:0, 22:0, 24:0); Σ Monounsaturated FA, total monounsaturated fatty acids (including 14:1, 16:1, 18:1, 20:1, 22:1, 24:1); Σ n-6, total n-6 fatty acids (including 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6); Σ n-3, total n-3 fatty acids (including 18:3n-3, 18:4n-3, 20:5n-3; 22:5n-3, 22:6n-3); Σ LCPn-6, total n-6 fatty acids of carbon chain length ⩾ C_{20} (including 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:5n-6); Σ LCPn-3, total n-3 fatty acids of carbon chain length ⩾ C_{20} (including 20:5n-3, 22:5n-3, 22:6n-3).

TABLE 3

Effect of Dietary Fat Composition on Fatty Acid Composition of Liver Lipid Classes[a]

Diet	Σ Saturated FA	Σ Monounsaturated FA	Σ n-6	Σ n-3	Σ LCPn-6	Σ LCPn-3
			(% wt of total fatty acids)			
Triglycerides						
Sow-fed	37.4 ± 2.2	38.5 ± 1.8	20.8 ± 0.5	3.3 ± 0.3	6.4 ± 0.8	1.8 ± 0.2
A	34.2 ± 1.6^{a,x}	18.4 ± 0.5^{a,x}	42.8 ± 1.8^{a,x}	4.6 ± 0.2^{a}	6.6 ± 0.7	1.6 ± 0.2^{a,b}
B	24.3 ± 0.7^{b,x}	37.2 ± 1.3^{b}	35.6 ± 1.6^{b,x}	3.0 ± 0.2^{b}	5.2 ± 0.6	0.9 ± 0.1^{a,x}
C	18.1 ± 0.5^{c,x}	47.8 ± 0.9^{c,x}	29.4 ± 1.0^{c,x}	4.7 ± 0.5^{a}	5.4 ± 0.5	1.8 ± 0.3^{b}
D	24.8 ± 1.2^{b,x}	44.0 ± 0.4^{d,x}	27.7 ± 0.9^{c,x}	3.6 ± 0.4^{a,b}	5.6 ± 0.5	1.4 ± 0.2^{a,b}
Phospholipids						
Sow-fed	41.0 ± 0.6	11.9 ± 0.7	36.4 ± 0.5	10.4 ± 0.4	22.7 ± 0.5	10.2 ± 0.4
A	42.3 ± 0.3^{a}	6.2 ± 0.2^{a,x}	42.8 ± 0.4^{a,x}	8.5 ± 0.5^{a,c,x}	20.0 ± 0.6^{a,b,x}	8.0 ± 0.5^{a,x}
B	38.6 ± 0.3^{b,x}	12.6 ± 0.3^{b}	40.2 ± 0.2^{b,x}	8.2 ± 0.2^{a,x}	20.3 ± 0.4^{a,b,x}	7.8 ± 0.2^{a,x}
C	36.4 ± 0.8^{c,x}	17.1 ± 0.8^{c,x}	36.7 ± 0.4^{c}	9.8 ± 0.4^{b,c}	19.1 ± 0.3^{a,x}	9.2 ± 0.4^{a,b}
D	38.8 ± 0.4^{b,x}	13.5 ± 0.4^{b}	37.7 ± 0.3^{c}	10.0 ± 0.2^{b}	21.8 ± 0.7^{b}	9.7 ± 0.2^{b}
Cholesteryl esters						
Sow-fed	25.8 ± 1.1	44.5 ± 2.2	27.8 ± 1.5	1.9 ± 0.3	5.2 ± 0.7	0.5 ± 0.0
A	26.6 ± 1.9^{a,b}	16.9 ± 1.0^{a,x}	54.1 ± 2.7^{a,x}	2.3 ± 0.2^{a,c}	4.0 ± 0.5	0.8 ± 0.2^{a,c}
B	22.2 ± 0.8^{a}	31.6 ± 1.6^{b,x}	44.4 ± 1.2^{a,c,x}	1.8 ± 0.1^{a}	3.1 ± 0.3	0.2 ± 0.1^{a}
C	37.7 ± 4.0^{b,x}	30.8 ± 1.2^{b,x}	27.6 ± 3.1^{b}	3.9 ± 0.6^{b,x}	4.0 ± 0.9	1.2 ± 0.2^{b,c,x}
D	33.9 ± 3.1^{b}	27.8 ± 1.1^{b,x}	34.8 ± 3.0^{b,c}	3.6 ± 0.3^{b,c,x}	4.6 ± 0.4	1.0 ± 0.1^{b,c}

[a]Values are means ± SE for five (sow-fed) or six (formula) animals per group. Values within groups A, B, C and D without a common superscript (a, b, c or d) are significantly different from other formula groups; x, significantly different from the sow-fed group ($P < 0.05$, corrected with Bonferroni factor). The abbreviations Σ Saturated FA, Monounsaturated FA, n-6, n-3, LCPn-6 and LCPn-3 are as listed in Table 2.

TABLE 4

Effect of Dietary Fat Composition on Fatty Acid Composition of Kidney Lipid Classes[a]

Diet	Σ Saturated FA	Σ Monounsaturated FA	Σ n-6	Σ n-3	Σ LCPn-6	Σ LCPn-3
			(% wt of total fatty acids)			
Triglycerides						
Sow-fed	49.7 ± 0.9	37.0 ± 1.2	12.3 ± 0.6	1.0 ± 0.1	3.3 ± 0.3	0.5 ± 0.1
A	42.4 ± 1.8^{a,x}	18.8 ± 0.4^{a,x}	36.2 ± 1.9^{a,x}	2.6 ± 0.1^{a,x}	8.6 ± 0.7^{a,x}	1.2 ± 0.1^{a}
B	30.6 ± 0.5^{b,x}	37.6 ± 0.7^{b}	30.0 ± 0.5^{b,x}	1.8 ± 0.1^{b,x}	6.3 ± 0.3^{b}	0.8 ± 0.1^{b}
C	23.2 ± 1.0^{c,x}	50.5 ± 0.9^{c,x}	23.7 ± 0.5^{c,x}	2.6 ± 0.1^{a,x}	4.1 ± 0.3^{c}	0.6 ± 0.1^{b,c}
D	35.3 ± 0.4^{d,x}	43.0 ± 0.7^{d,x}	19.7 ± 0.8^{c,x}	2.1 ± 0.1^{b,x}	3.4 ± 0.6^{c}	0.4 ± 0.1^{c}
Phospholipids						
Sow-fed	38.0 ± 0.6	20.2 ± 0.5	37.4 ± 0.3	4.4 ± 0.1	23.4 ± 0.2	4.2 ± 0.1
A	37.3 ± 0.6^{a}	13.9 ± 0.2^{a,x}	44.6 ± 0.8^{a,x}	4.2 ± 0.1^{a}	21.5 ± 0.8^{a}	3.4 ± 0.1^{a,x}
B	33.9 ± 0.9^{b,x}	22.9 ± 0.4^{b,x}	39.1 ± 1.3^{b}	4.1 ± 0.4^{a}	19.0 ± 0.8^{b,x}	3.4 ± 0.3^{a,x}
C	27.2 ± 0.5^{c,x}	30.6 ± 0.7^{c,x}	36.6 ± 0.9^{c}	5.4 ± 0.3^{b,x}	19.3 ± 0.6^{b,x}	4.5 ± 0.2^{b}
D	30.1 ± 0.5^{d,x}	25.8 ± 0.4^{d,x}	38.8 ± 1.4^{b,c}	5.2 ± 0.2^{b,x}	21.9 ± 0.8^{a}	4.5 ± 0.2^{b}

[a]Values are means ± SE for five (sow-fed) or six (formula) animals per group. Values within groups A, B, C and D without a common superscript (a, b, c or d) are significantly different from other formula groups; x, significantly different from the sow-fed group ($P < 0.05$, corrected with Bonferroni factor). The abbreviations Σ Saturated FA, Monounsaturated FA, n-6, n-3, LCPn-6 and LCPn-3 are as listed in Table 2.

TABLE 5

Effect of Dietary Fat Composition on Fatty Acid Composition of Brain Total Lipid[a]

Diet	Σ Saturated FA	Σ Monounsaturated FA	Σ n-6	Σ n-3	Σ LCPn-6	Σ LCPn-3
			(% wt of total fatty acids)			
Sow-fed	46.0 ± 0.3	24.7 ± 0.5	19.2 ± 0.1	10.1 ± 0.3	17.8 ± 0.1	10.1 ± 0.3
A	46.6 ± 0.3^{a}	22.7 ± 0.3^{a,x}	20.5 ± 0.2^{a,x}	10.2 ± 0.1	18.5 ± 0.2	10.1 ± 0.1
B	46.2 ± 0.3^{a,b}	23.8 ± 0.2^{a,b}	20.0 ± 0.3^{a,b}	10.1 ± 0.2	18.1 ± 0.3	10.1 ± 0.2
C	45.1 ± 0.3^{b}	24.7 ± 0.4^{b}	19.3 ± 0.4^{b}	10.9 ± 0.2	17.7 ± 0.3	10.8 ± 0.2
D	45.7 ± 0.3^{a,b}	23.7 ± 0.3^{a,b}	19.9 ± 0.1^{a,b}	10.8 ± 0.2	18.3 ± 0.1	10.7 ± 0.2

[a]Values are means ± SE for five (sow-fed) or six (formula) animals per group. Values within groups A, B, C and D without a common superscript (a or b) are significantly different from other formula groups; x, significantly different from the sow-fed group ($P < 0.05$, corrected with Bonferroni factor). The abbreviations Σ Saturated FA, Monounsaturated FA, n-6, n-3, LCPn-6 and LCPn-3 are as listed in Table 2.

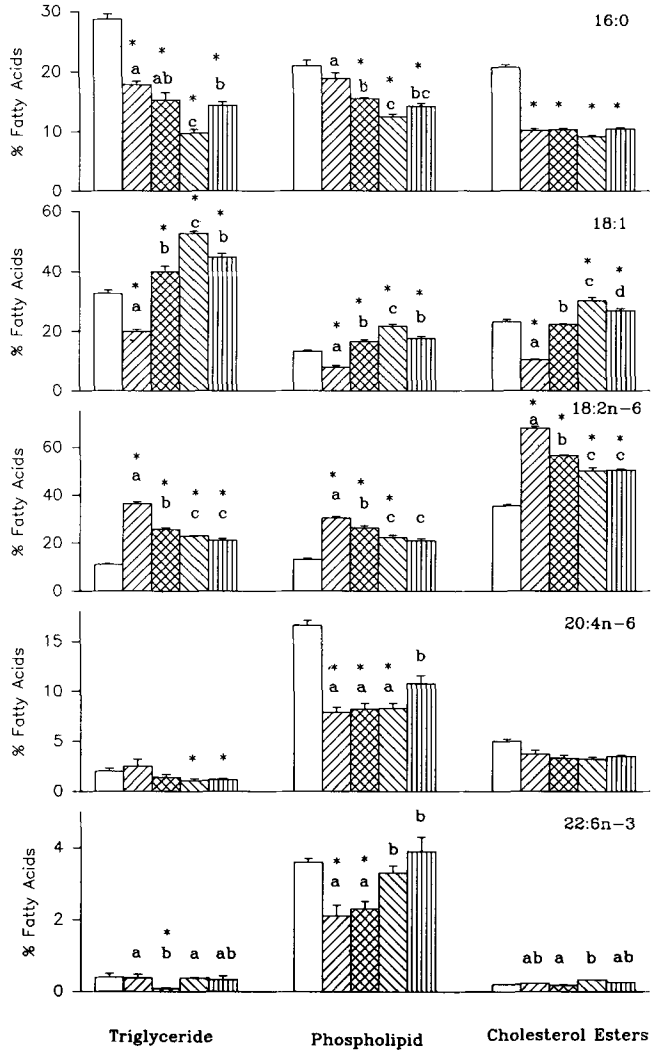

FIG. 1. The effect of formula fat composition, or sow milk feeding on the percent composition of 16:0, 18:1, 18:2n-6, 20:4n-6 and 22:6n-3 in plasma triglyceride, phospholipid and cholesterol ester fractions. Values are means ± SE for five (sow-fed) or six (formula groups) animals per group. Formula groups without a common superscript are significantly different from each other, or with the superscript *, are significantly different from the sow-fed group ($P < 0.05$, corrected with the Bonferroni factor). Groups (as defined in the text) are identified as: sow-fed (open bars), formula A (right-hatched bars), formula B (cross-hatched bars), formula C (left-hatched bars) and formula D (vertical line bars).

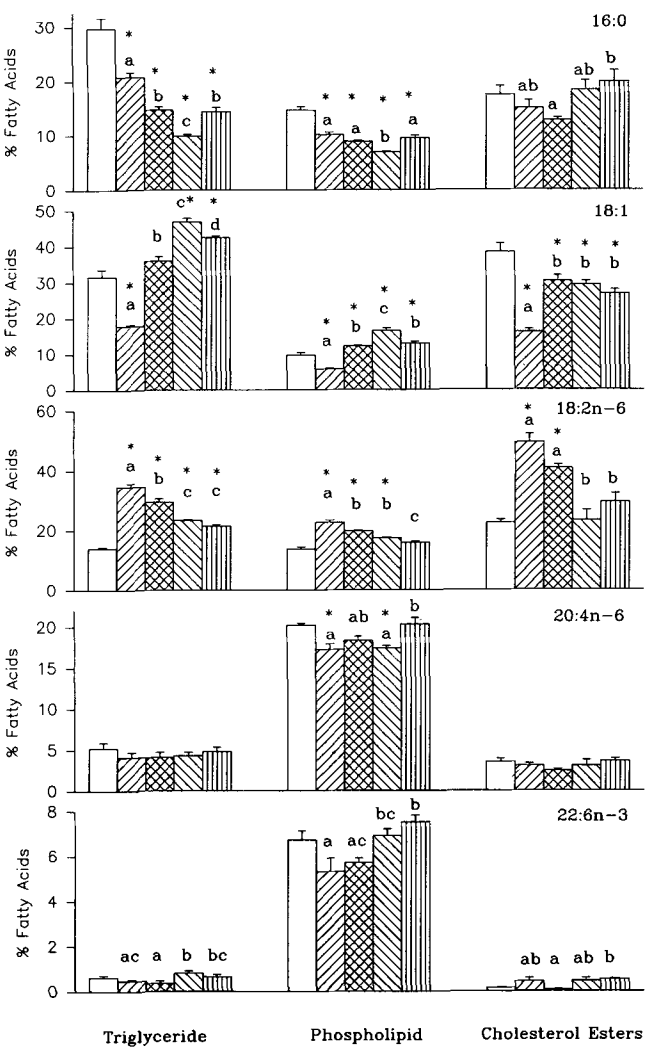

FIG. 2. The effect of formula fat composition or sow milk feeding on the percentage of 16:0, 18:1, 18:2n-6, 20:4n-6 and 22:6n-3 in liver triglyceride, phospholipid and cholesterol ester fractions. Values are means ± SE for five (sow-fed) or six (formula groups) animals per group. Formula groups without a common superscript are significantly different from each other, or with the superscript *, are significantly different from the sow-fed group ($P < 0.05$, corrected with the Bonferroni factor). Groups (as defined in the text) are identified as: sow-fed (open bars), formula A (right-hatched bars), formula B (cross-hatched bars), formula C (left-hatched bars) and formula D (vertical line bars).

Σ n-3-Σ LCPn-3 in the results in Tables 2-4. No significant differences in brain total lipid were found between formula and sow-fed animals, with the exception of lower Σ monounsaturated and higher Σ n-6 fatty acids in piglets fed formula A (Table 5).

Plasma lipids. The increase in dietary 18:1 at the expense of MCFA in formula B compared to A resulted in significantly higher levels of 18:1 and lower percentage of 18:2n-6 in the piglet plasma TG, PL and CE, even though the formulas had similar amounts of 18:2n-6 (Fig. 1). The percentage of 20:4n-6 in plasma TG, PL and CE, and the percentage of 22:6n-3 in PL and CE, however, were similar in piglets fed these two formulas. In contrast, the TG percentage of 22:6n-3 was lower in piglets fed formula B than formula A (Fig. 1). These changes are reflected in the sum (Σ) of the percentage of monounsaturated, n-6 and n-3 fatty acid values in Table 2. The significantly lower Σ saturated fatty acids in plasma PL of piglets fed formula B compared to A (Table 2) was predominantly due to the lower percentage of 16:0 (Fig. 1).

Piglets fed formula D had lower levels of 18:1 in plasma TG, PL and CE but higher levels of 20:4n-6 in PL than piglets fed formula C, containing similar amounts of 18:1, 18:2n-6 and 18:3n-3 but with MCFA rather than ICFA (Fig. 1). The Σ saturated fatty acids (Table 2) in all the lipid classes and the percentage of 16:0 in TG (Fig. 1) were also higher in piglets fed formula D *vs.* formula C.

Formulas B and D had similar amounts of 18:1 and 18:3n-3, but 25.6 and 15.6% 18:2n-6 with n-6/n-3 ratios of 8.0 and 4.9, respectively. Piglets fed formula D, with the

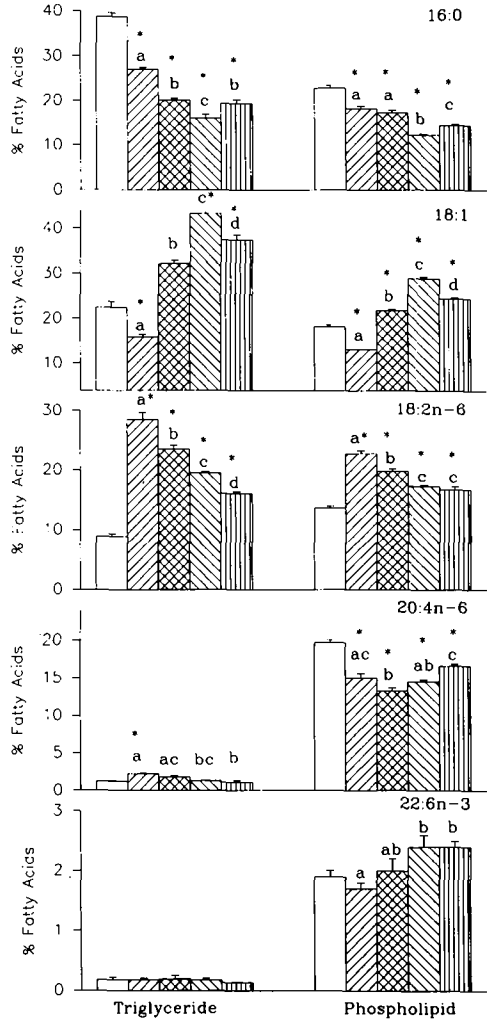

FIG. 3. The effect of formula fat composition or sow milk feeding on the percentage of 16:0, 18:1, 18:2n-6, 20:4n-6 and 22:6n-3 in kidney triglyceride and phospholipid fractions. Values are means ± SE for five (sow-fed) or six (formula groups) animals per group. Formula groups without a common superscript are significantly different from each other, or with the superscript *, are significantly different from the sow-fed group ($P < 0.05$, corrected with the Bonferroni factor). Groups (as defined in the text) are identified as: sow-fed (open bars), formula A (right-hatched bars), formula B (cross-hatched bars), formula C (left-hatched bars) and formula D (vertical line bars).

lower 18:2n-6 and n-6/n-3 ratio, had lower levels of 18:2n-6 in the plasma TG, PL and CE and higher 20:4n-6 and 22:6n-3 in PL than piglets fed formula B (Fig. 1).

Liver lipids. Analysis of the fatty acid composition of liver from piglets fed formula A containing 11% 18:1 showed significantly lower levels of 18:1 in the TG, PL and CE but higher 18:2n-6 and Σ saturated fatty acids in TG and PL than in piglets fed formula B containing 36% 18:1 (Fig. 2 and Table 3). No significant differences were found in the % 20:4n-6, 22:6n-3 (Fig. 2) or n-6 or n-3 LCP (Table 3) between these two groups. Feeding formula with saturated fatty acids as ICFA (formula D) rather than MCFA (formula C), with similar levels of 18:1, 18:2n-6 and 18:3n-3, resulted in significantly lower percentage of 18:1 and 18:2n-6 in PL and 18:1 in TG. Levels of 16:0 and 20:4n-6 in PL, and 16:0 in TG, however, were higher (Fig. 2). Although the fatty acid composition of the liver CE was similar between piglets fed formulas C and D (Table 3 and Fig. 2), the higher n-6/n-3 ratio of 8.0 in formula B compared to 4.9 in formula D was associated with significantly higher levels of 18:2n-6 and lower 22:6n-3 (Fig. 2) and total saturated fatty acids (Table 3), in liver CE.

Kidney lipids. Cholesteryl ester levels were low in the kidney, and were not analyzed in the present study. The higher dietary intake of 18:1 in piglets fed formula B compared to A was associated with significantly higher levels of 18:1 in kidney TG and PL, lower percentage of 16:0 and 18:2n-6 in TG (Fig. 3) and lower Σ saturated fatty acids (Table 4), percentage of 18:2n-6 and percentage of 20:4n-6 (Fig. 3) in PL.

Feeding formula D containing ICFA compared to formula C containing MCFA resulted in significantly higher levels of 16:0 and 20:4n-6 and lower levels of 18:1 in kidney PL. Levels of 16:0 were higher and 18:1 and 18:2n-6 lower in TG despite the similar quantities of 18:1, 18:2n-6 and 18:3n-3 in these two diets (Fig. 3).

The kidney lipids of piglets fed formula B containing 36% 18:1 and with an n-6/n-3 ratio of 8.0 had significantly more 18:2n-6 and less 18:1 in TG and PL, and more 16:0 and less 20:4n-6 in PL (Fig. 3) than piglets fed formula D with 39% 18:1 and an n-6/n-3 ratio of 4.9.

Brain lipids. There were no significant differences in the fatty acid composition of brain total lipid (primarily phospholipid) between piglets fed formulas A and B containing 11 and 36% 18:1, or formulas C and D containing MCFA and ICFA, respectively (Table 5). These findings are in contrast to analyses of plasma, liver and kidney, in which (i) the monoenoic fatty acid content of the diet (11% in formula A and 36–41% in formulas B–D) and (ii) the lower 18:2n-6 content of formulas A and B *vs.* C and D, were reflected in TG, PL and CE fatty acids. Similarly, feeding diets B and D with n-6/n-3 ratios of 8.0 and 4.9 did not influence brain fatty acid composition (Table 5).

DISCUSSION

Results from these studies comparing four groups of formula fed and a group of sow-fed piglets show that (i) substitution of monoenoic fatty acids for MCFA, while maintaining diet 18:2n-6 and 18:3n-3 levels, influences the metabolism of 18:2n-6 by altering the content of 18:2n-6, and the ratio of 18:2n-6 to its desaturation product 20:4n-6, in plasma and organ lipid classes, (ii) the dietary source of saturated fatty acid—either MCFA from MCT oil or ICFA from coconut oil—when fed with similar levels of 18:1, 18:2n-6 and 18:3n-3, selectively alters the percentage of 18:1, 18:2n-6 and 20:4n-6 in plasma, liver and kidney TG, PL and CE, (iii) the chain length of dietary saturated fatty acids (MCFA *vs.* ICFA) is an important determinant of tissue 20:4n-6 levels, (iv) dietary percentage of 18:2n-6, and/or n-6/n-3 ratio may influence plasma and organ PL 22:6n-3, (v) manipulation of saturated and monounsaturated fatty acids in formula fat blends can change the plasma and tissue levels of essential fatty acids and their LCP products, and (vi) in contrast to plasma, liver and kidney lipids, brain total lipid fatty acid composition was not influenced by dietary 18:1 content, MCFA *vs.* ICFA or n-6/n-3 ratio with the formulas used in the present studies.

The competition for chain elongation and desaturation among oleic (18:1), linoleic (18:2n-6) and linolenic (18:3n-3) acids (8) and specificity for incorporation of fatty acids into different lipid classes (20–23) are well known. The complexity of fatty acid metabolism and the limitations in experimental design imposed by available oil sources, however, often make it difficult to interpret the effect of a specific dietary fatty acid on the tissue fatty acid response. Little information is currently available on the effect of dietary 18:1 on the incorporation of other dietary fatty acids into tissue lipids. In agreement with previous studies (24,25), the diets containing high levels of 18:1 led to an increase in plasma and tissue percentage of 18:1, and a concomitant decrease in percentage of saturated fatty acids. Results from several studies suggest that 18:1 may have unique or different effects on fatty acid metabolism when compared with saturated or polyunsaturated fatty acids. For example, stimulation of Δ5 desaturase activity following triolein administration (26), avid incorporation of 18:1 into liver TG, and higher rates of secretion of 18:1 than 18:2n-6 or 18:3n-3 in liver very low density lipoprotein (VLDL) (21,27,28) have all been shown. Dietary oleic acid may also result in higher liver cholesterol levels than polyunsaturated fatty acids such as 18:2n-6 (29). It is reasonable to assume, therefore, that the diet 18:1 content may influence or determine, at least in part, the metabolism of other dietary fat components.

The present studies in which piglets were fed diets with constant levels of 18:2n-6 but different levels of 18:1 (and MCFA) show that increased tissue levels of 18:1 are accompanied by decreased 18:2n-6 in the plasma, liver and kidney TG, PL and CE. This effect does not appear to be related to the formula content of MCFA, as no correlation between dietary MCT content and tissue 18:2n-6 was found among groups A, B and C containing 20–40% (w/w of diet fat) MCT oil. Recent studies from this laboratory (30) reported that the dietary intake of 18:3n-3 from formula influenced the amount of 18:1 in piglet plasma and liver PL, but that the effect also was influenced by the 18:2n-6 content of the formula. In the latter studies, the formula 18:2n-6 was adjusted by changing the 18:1 content. The results of the present studies, in which the 18:3n-3 content of the formula fat blends was held constant (Table 1), suggest the interaction of diet 18:1 and 18:2n-6 is the predominant determinant of plasma and tissue levels of these fatty acids.

Differences in plasma, liver and kidney PL 18:2n-6, but not 20:4n-6, were found among piglets fed formulas providing similar amounts of 18:2n-6. The increased 18:1 and decreased 18:2n-6 in the PL of piglets fed diet B, containing 36% 18:1, compared to diet A, containing 11% 18:1, could possibly be explained by competitive replacement of 18:1 for 18:2n-6 in pathways of acylation. High levels of 18:1 in a formula containing ICFA (D; 39% 18:1) compared to MCFA (B and C; 36–41% 18:1) also resulted in lower tissue PL and/or TG 18:2n-6. This suggests that the acylation, and potentially also the oxidation, of 18:1 and 18:2n-6 are influenced by the diet saturated fatty acid chain length.

The maintenance of plasma and tissue PL 20:4n-6, despite the significant differences in PL 18:2n-6, among piglets fed formula varying in saturated and monounsaturated fatty acid content is noteable. These results provide evidence that tissue PL levels of 20:4n-6 are tightly regulated and not necessarily influenced by dietary intakes of 18:2n-6 which are well in excess of minimum requirements. In this regard, a specific arachidonyl-CoA synthetase has been identified (31). It is suggested that the diet fatty acid composition influences the metabolism of the dietary 18:2n-6, primarily by changing the proportion of 18:2n-6 directly acylated into tissue lipid, or potentially the amount oxidized, rather than by altering the amount entering the microsomal desaturation-elongation pathway. It is possible, however, that recycling of 20:4n-6 during turnover reactions could maintain tissue 20:4n-6 levels.

Increased tissue levels of 22:6n-3 following feeding with diets high in 18:1 have been reported by Periago *et al.* (25). The studies reported here provide further evidence that the diet 18:1 content may influence the desaturation-elongation of 18:3n-3 to 22:6n-3 (Figs. 1–3). The difference in plasma and tissue PL percentage of 22:6n-3 (group C = D > A = B), despite similar dietary intake of 18:3n-3, could possibly be explained by the lower intake of 18:2n-6 and lower n-6/n-3 ratio of formulas C and D, thus reducing the competition between 18:2n-6 and 18:3n-3 for desaturation. Data published by Lee *et al.* (32), however, do not support an effect on tissue lipid levels of 20:4n-6 and 22:6n-3 due to changes in the n-6/n-3 ratio within the range used in the piglet formulas.

The present studies compared the effects on tissue fatty acids of feeding MCT containing primarily fatty acids of chain length 8:0 and 10:0, and coconut oil containing primarily fatty acids of chain length 12:0 and 14:0 in similar amounts to that used in infant formulas. The physical and biochemical properties of 12:0 and 14:0 are intermediate between fatty acids of chain length >14:0 and ≤10:0. However, 12:0, unlike 8:0 or 10:0, is accepted as substrate by *sn*-glycero-3-phosphate and 1-acyl-*sn*-glycero-3-phosphate acyltransferases and is secreted from liver in VLDL TG (28). Deposition of 12:0 in adipose tissue TG at rates comparable to that expected for dietary LCFA has also been reported (33,34). In contrast, 8:0 and 10:0 do not appear to be used as substrate for phospholipid synthesis (7,35) and are incorporated only slowly into hepatic TG *via* 1,2-diacyl-*sn*-glycerol acyltransferase (35). The saturated fatty acids of coconut oil and MCT oils, therefore, can be expected to be handled differently. In addition, oxidative flux of MCFA far exceeds that of longer chain fatty acids (36), such that lesser amounts of these fatty acids are available for incorporation into lipids. Differences in route of absorption, direct transport of MCFA as unesterified fatty acids *via* the portal vein *vs.* transport of longer chain fatty acids (C > 14) in chylomicron TG *via* the lymph, may also be expected to alter processes such as intestinal apoprotein expression (37–39), the composition and size of the chlyomicron (37) and subsequent processing, including the activity of hepatic and lipoprotein lipase on chylomicron and VLDL TG (40,41). Dietary MCFA therefore, by nature of its rapid and almost complete oxidation, and specific metabolism, may be expected to also influence the oxidation, acylation and further metabolism, such as elongation/desaturation, of concurrently fed fatty acids. For example, despite similar levels of 18:1 in the fat blends of formulas C and D, animals fed formula C with MCFA had consistently higher levels of 18:1 in TG, PL and CE than animals fed formula D with ICFA. This could be explained by higher endogenous 18:1 synthesis, including Δ9 desaturase activity, in piglets fed

MCFA. Alternatively, preferential oxidation of 8:0 and 10:0 from MCT could spare longer chain dietary fatty acids, including 18:1, from oxidation for energy. The latter hypothesis is compatible with the higher levels of 18:2n-6 found in the tissues of piglets fed formulas containing MCFA compared to ICFA.

Clinical studies on fatty acid nutrition often include measures of the fatty acid composition of plasma and RBC PL (10–14). The present studies indicate that while these measures generally reflect liver and kidney lipids, they are not an accurate reflection of brain lipid, which appears to maintain a very stable fatty acid profile over a wide range of dietary fatty acid intakes. It seems that much more information is needed regarding the effects of various dietary fatty acids on the processes involved in tissue structural lipid formation before the dietary fatty acid composition required for optimal development and function of different organs can be defined.

CONCLUSION

The results of these studies demonstrate interaction between dietary nonessential saturated and monounsaturated fatty acids and metabolism of n-6 and n-3 fatty acids. This is relevant for future development of infant formulas, especially those for very premature infants with concomitant complications of immaturity, particularly those related to fat digestion and absorption. A major emphasis in recent years has been on the potential need for a dietary supply of carbon chain 20 and 22 n-6 and n-3 fatty acids (9,10,12,13), particularly 20:4n-6 and 22:6n-3. Our data indicate that the saturated and monounsaturated fatty acids supplied in the diet with 18:2n-6 and 18:3n-3 can affect the metabolic handling of the n-6 and n-3 fatty acids.

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Effects of 17β-Estradiol and Starvation on Trout Plasma Lipoproteins

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Administering 17β-estradiol (E_2) to juvenile trout results in plasma hyperlipidemia and hyperlipoproteinemia associated with significant increases in the concentrations of triglycerides (TG), free cholesterol, phospholipids, free fatty acids and proteins, both postprandial and during starvation. TG undergo the greatest increase (9 times control level 96 h after feeding). The concentration differences between E_2-treated and control trout increase during starvation, primarily by progressive decreases in the concentrations of various lipids in controls. E_2-induced hypertriglyceridemia is mainly caused by an increase in the concentration of very low density lipoproteins (VLDL) during both the postprandial period (6 times control level at 24 h) and during starvation (15 times control level at 96 h); hyperlipoproteinemia lasts up to at least 7 d after the last feeding. E_2 treatment does not change the concentration of high density lipoproteins, but does increase plasma concentrations of a very high density lipoprotein, vitellogenin (VTG). In E_2-treated VLDL, cholesteryl esters are depleted while proteins are enriched. During the postprandial phase, the apolipoprotein (apo) profile of VLDL (d < 1.015 g/mL) is comparable in E_2-treated and control trout. Starvation of E_2-treated trout is accompanied by an enrichment in apo B_{240}, A-I and A-II. The secretion levels of TG and VLDL-TG, as determined *in vivo* by injecting Triton WR-1339 to starving animals, are significantly higher in E_2-treated trout than in controls. In trout, as in chicks, E_2 administration significantly increases the concentration and hepatic secretion of plasma VLDL independent of the nutritional status and the appearance of VTG in the plasma. This suggests the existence of similar mechanisms for the regulation of lipoprotein metabolism by estrogens in oviparous vertebrates.
Lipids 27, 1032–1041 (1992).

Sex steroids modify the risk of atherosclerosis by regulating the levels of plasma lipoproteins (1–3). Natural or induced changes in estrogen concentrations in various species have been shown to affect the production and catabolism of lipoproteins. The mechanisms involved in the action of estrogens on lipoprotein metabolism appear to be largely dose dependent (4,5).

Pharmacological doses of estrogens in mammals stimulate the number of low density lipoprotein (LDL) receptors in rat (6,7) and rabbit liver (8) and in the HepG2 human hepatocarcinoma cell line (9). In humans, estrogens reduce hepatic triglyceride lipase activity (10–12) and increase the concentrations of plasma high density lipoproteins (HDL) (10–13) and very low density lipoproteins (VLDL) (11,14). In humans, estrogens also stimulate apolipoprotein synthesis by HepG2 cells (15,16). Plasma concentration and hepatic secretion of VLDL have been shown to increase in rats after treatment with ethinyl estradiol (17–19).

Vitellogenesis is characterized by the accumulation of large reserves of yolk by growing oocytes. Yolk proteins and lipids are derived from plasma precursors, primarily lipoproteins (20). Vitellogenesis in birds results essentially from oocyte uptake of two classes of different density lipoproteins, VLDL and vitellogenin (VTG), a very high density lipoprotein. Hepatic synthesis of these two macromolecular complexes is stimulated dramatically by estrogens during sexual maturation of females or during estrogen treatment of males or immature females (21,22). The molecular mechanisms of the regulation of lipoprotein and apolipoprotein syntheses have been studied in birds, especially in chicks (4,22,23). Estrogen-induced hyperlipidemia in this species is characterized by a pronounced increase in VLDL levels, a smaller increase in LDL levels and a decrease in HDL levels (24–26). VLDL possess only two major apolipoproteins (apo), apo B and apo VLDL-II; the latter is synthesized by the liver under the strict control of estrogens (22). The presence of apo VLDL-II results in a considerable decrease of VLDL sensitivity toward lipoprotein lipase (LPL) (27,28). These VLDL are then incorporated in growing oocytes by an apo B-specific surface receptor (29) which is identical to the VTG receptor (30).

VTG has been detected in the blood of a large number of fish species during their normal reproduction cycle or in response to estrogen stimulation (31). Its hepatic synthesis can be induced by 17β-estradiol (E_2) (32) and is correlated with the circulating levels of this hormone during vitellogenesis (33,34).

No data exist on the possible role of estrogens in the metabolic regulation of other lipoprotein classes in fish. The aim of this work was to investigate the combined effects of nutritional status and brief E_2 treatment in juvenile trout on the qualitative and quantitative changes in the different lipoprotein classes of the plasma. In particular, we have shown that in trout, as in chicks and rats, E_2 administration causes a significant increase in the concentration and secretion of plasma VLDL, independent of nutritional status.

MATERIALS AND METHODS

Plasma. Juvenile rainbow trout, *Oncorhynchus mykiss* (formerly *Salmo gairdneri*) were raised at constant temperature (13 ± 0.5°C) and nourished *ad libitum* three times a week with granules (Aqualim, Nersac, France) containing 8% lipids. The trout were anesthetized with ethylenglycol monophenyl ether (0.3 mL/L). Blood was removed over ethylenediaminetetraacetic acid (EDTA) disodium salt and NaN_3 (3 and 0.15 mg/mL blood) dissolved in 0.15 M NaCl, final pH 7.4, by cardiac punc-

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Abbreviations: Apo, apolipoprotein; BSA, bovine serum albumin; BW, body weight; CE, cholesteryl esters; E_2, 17β-estradiol; EDTA, ethylenediaminetetraacetic acids; FC, free cholesterol; FFA, free fatty acids; HDL, high density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; LW, liver weight; LPL, lipoprotein lipase; LSI, liver somatic index; PL, phospholipids; SDS, sodium dodecylsulfate; TC, total cholesterol; TG, triglycerides; TGSR, triglyceride secretion rates; VLDL, very low density lipoproteins; VTG, vitellogenin.

ture with a fine catheter (diameter 0.58 mm) and kept at 4°C throughout the procedure. Plasma was obtained by centrifugation (3000 × *g*, 10 min).

17β-Estradiol and fasting treatments. A graphic representation of our study design is shown in Figure 1. Animals were anesthetized and injected intraperitoneally with E_2 (7 mg/kg body wt) on days 0, 4, and 8; the injection volume was 1 μL/g body wt. E_2 was first dissolved in anhydrous ethanol and then in cottonseed oil (1:4, vol/vol). Controls were injected with ethanol and cottonseed oil without E_2. This treatment was selected to produce a rapid elevation in E_2 and VTG plasma levels, to the same range found during exogenous vitellogenesis in females (33–37). During the treatment there was no mortality, and the trout were still feeding.

Two-year-old juvenile trout of both sexes, weighing 278 ± 61 g (± SD, n = 26) from controls and the E_2-treated group were starved for 3 d and then fed *ad libitum* on day 10 of the treatment. The effects of starvation with or without estrogen treatment on plasma lipoprotein profiles were evaluated with serial blood samples taken until 7 d after the last meal. The blood volumes collected were lower than 2% of the whole body blood volume. Quantitative and chemical analysis of plasma lipids, proteins and lipoproteins in the two groups were performed with blood samples taken in series at 24, 96 and 154 h after feeding. The blood volumes punctured were lower than 10% of the total whole body blood volume. Alimentary assimilation is slower in fish than in mammals (31).

At the conclusion of the experiments (day 17, see Fig. 1) fasted fish were anesthetized and killed. Body (BW) and liver weights (LW) were determined to calculate the liver somatic index [LSI = (LW/BW) × 100].

Lipoprotein fractionation and analysis. A density gradient ultracentrifugation procedure previously described (38) was used with modifications for the fractionation of whole plasma lipoprotein classes and proteins. Discontinuous six-step density gradients were prepared with NaBr solutions, containing 0.05% EDTA (pH 7.4). Successive densities were (from top to bottom of tube): 1.006 g/mL (1.1 mL); 1.019 g/mL (2.5 mL); 1.063 g/mL (3.5 mL); 1.210 g/mL (2.5 mL); 1.310 g/mL (plasma at 1.310 g/mL, 0.55 or 0.01 mL, adjusted to this density with solid NaBr, and completed with a 1.310 g/mL NaBr solution to give a final volume of 1 mL); and 1.386 g/mL (0.8 mL). Six percent of the plasma volume is assumed to consist of macromolecules, and the remaining 94% is equivalent to a salt solution of density 1.015 g/mL. Instead of the adjusted plasma sample, control gradients were prepared using a NaBr solution of density 1.015 g/mL adjusted to 1.310 g/mL with solid NaBr. The gradients were then placed in a Beckman (Palo Alto, CA) SW 41-Ti swinging bucket rotor (average radius 110.2 mm) and centrifuged at 180,000 × *g* for 24 h at 10°C in a Beckman L8-70 ultracentrifuge, without braking at the end of the run.

The centrifuge tube containing the separated lipoproteins was punctured at the bottom and connected to the fractionation system filled with a 1.386 g/mL NaBr solution. The lipoprotein profile was recorded by continuously monitoring absorbance at 280 nm with a UV monitor (LKB 2238 Uvicord SII, LKB, Bromma, Sweden) as the lipoproteins were pumped from the centrifuge tube (collection speed 0.6 mL/min, chart speed 10 mm/min). One arbitrary unit of absorbance was taken as the optical

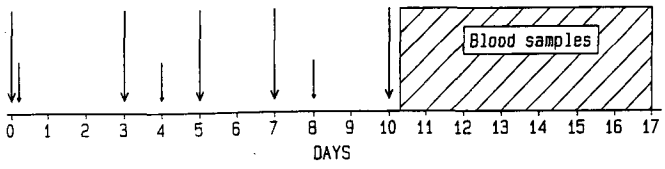

FIG. 1. Graphic representation of the study design. The small arrows indicate when 17β-estradiol or vehicle alone was intraperitoneally injected. The large arrows indicate when trout were nourished. The shaded area represents the period of blood sampling.

density of a 1 mg/mL solution of bovine serum albumin (BSA; Cohn fraction V, Sigma Chemical Co., St. Louis, MO). At the end of the fractionation, distilled water was carefully deposited onto the upper layer of the tube until VLDL was completely eluted. Fractions were collected with a fraction collector. Quantitative lipoprotein profiles were determined based on total lipoprotein concentration and the volume of the combined tube fractions (60 fractions, mean volume 300 μL). For each fraction, the lipoprotein concentration was calculated as follows: total lipoproteins = cholesteryl esters (CE) + free cholesterol (FC) + triglycerides (TG) + phospholipids (PL) + proteins. Density regions used were VLDL (VLDL + chylomicrons at + 24 h after feeding), d < 1.015 g/mL; LDL (intermediate density lipoproteins (IDL) + LDL), 1.015 < d < 1.085 g/mL; HDL, 1.085 < d 1.210 g/mL; VTG, (presence of VTG only for estrogen-treated fish), 1.210 < d < 1.310 g/mL; proteins, d > 1.310 g/mL. The concentrations were expressed as mg/dL plasma after correction for dilution introduced by the anticoagulant. The recovery after the entire procedure was 86.03 ± 4.32% for CE, 92.94 ± 2.93% for FC, 95.58 ± 3.24% for TG, 84.57 ± 3.09% for PL, and 88.73 ± 2.19% for proteins (n = 14).

The apolipoprotein compositions of the major classes of trout lipoproteins have recently been described (31,38, 39). Apolipoproteins were electrophoresed under reducing conditions in sodium dodecylsulfate (SDS)/glycerol/polyacrylamide slab gels using a linear gradient of 3.5–15% polyacrylamide and 8–12% glycerol (39). The relative molecular mass (Mr) values of trout apolipoproteins were determined as previously described (39) by comparison with simultaneously run proteins of known Mr (MW-SDS-70L and MW-SDS-200 molecular weight marker kits from Sigma Chemical Co.). Gels were subsequently fixed and stained using a highly sensitive Coomassie Blue G-250 procedure (40).

The size distribution of VLDL isolated 96 h after feeding was monitored by electron microscopy (80 kV) after dual staining of the particles (41). VLDL were diluted first with saline EDTA, mixed with 1:1 (vol/vol) 4% OsO_4, and fixed for 2 min. The fixed and positively stained particles were then negatively stained with 1% neutral phosphotungstic acid in the presence of 0.1% sucrose on a Formvar/carbon grid (Balzers, Selles S/Cher, France).

Chemical analysis. The lipid compositions of the lipoprotein fractions and of plasma were estimated using commercially available enzymatic kits from Boehringer-Mannheim (Meylan, France) for FC, total cholesterol (TC) and free fatty acids (FFA), and from Wako-Unipath (Dardilly, France) for PL and TG. The assays were performed as described elsewhere (42,43). Protein concentrations were determined by a modified Lowry assay (44) using BSA

as standard. The amount of CE was calculated using the formula CE = 1.7 × (TC−FC), using 288 as the average molecular weight of the trout plasma fatty acids (45). The amount of total lipids (TL) in plasma were calculated from the expression TL = CE + FC + PL + TG + FFA (46). Concentrations in plasma were expressed as mg/100 mL plasma after correction for the dilution introduced by the anticoagulant.

Ultrastructural study. The tissues were fixed for 1 h at 4°C in osmium tetroxide (OsO_4) buffered with sodium cacodylate (0.15 M, pH 7.3) and embedded in Epon 812. The lipids were preferentially contrasted by the OTO method (47). Thin sections of tissue were collected on gold grids and successively placed on 1% aqueous solution of thiocarbohydrazide (1 h at 50°C) and on 2% aqueous solution of osmium tetroxide (1 h at 50°C).

VLDL triglyceride secretion. To evaluate the effect of E_2 on TG and VLDL-TG secretion rates, trout were injected with Triton WR-1339 (Tyloxapol, Sigma Chemical Co.), a nonionic detergent which has been used widely as an indicator of VLDL secretion *in vivo* (25, 48–51). Eleven-month-old juvenile trout of both sexes, weighing 63.61 ± 10.3 g (±SD, n = 23) from controls and the E_2-treated group were starved for 96 h before Triton injection. Preliminary experiments indicated that adequate inhibition of TG removal occurs at 70 mg/100 g body wt or above. Animals were anesthetized, and Triton WR-1339 (25 g/dL in 0.9% NaCl, 70 mg/100 g body wt) was injected intracardialy at day 14 of the estrogen or control treatment (injection volume, 145 μL). For each trout, blood samples were taken intracardialy at 15 min (arbitrary zero time) and 3 h or 6 h after the Triton injection. The mean of TG secretion rates (TGSR) for each group, E_2-treated or control animals, was determined by the equation TGSR = 1/2 (TG1/T1 + TG2/T2) × PV (49). TG1 and TG2 are means augmentations of plasma TG concentrations (mg/dL) 3 and 6 h after Triton injection, and T1 and T2 are times between zero time and time of sampling after Triton injection. PV is the estimated whole body plasma volume (3.25 mL/100 g body wt) (52,53).

VLDL were isolated by micro-ultracentrifugation in an air-driven ultracentrifuge (Airfuge, Beckman, Palo Alto, CA) (54,55) from plasma of the same trout obtained 15 min (zero time) and 3 h after Triton injection. One hundred μL plasma was transferred to a centrifuge tube, layered gently with 75 μL of NaBr solution (d = 1.015 g/mL), and centrifuged at 100,000 rpm (30 psi) for 3 h in a fixed angle rotor (A-100/30). The air was refrigerated at 4°C using an air drying and cooling system (56). Thirty μL of top VLDL fraction was cut with a tube slicer specifically designed for the small tubes. TG values were determined in control and E_2-treated trout to evaluate the difference in VLDL output *in vivo* after Triton WR-1339 injection. Pure VLDL, not contaminated by other lipoprotein classes or plasma proteins, were isolated by this procedure (57). However, the volume collected here was too low for a complete recovery of the VLDL fraction (54,55).

Statistical methods. Data are presented as means ± SEM and were tested for statistical significance by analysis of variance followed by an evaluation employing Scheffe's multiple range test. LSI comparison was made by Student's *t*-test. Statistical evaluations of the results were performed using the Statistical Analysis System (SAS) (58). The *P* value chosen for statistical significance was 0.05.

RESULTS

Concentration of plasma lipid fractions and proteins. The effects of starvation on the concentration of different plasma lipids and proteins, with or without E_2 treatment, are shown in Table 1. Plasma samples were obtained from juvenile control or treated trout 24, 96 and 154 h after the last feeding.

E_2 treatment increased the concentrations of plasma lipids and proteins except CE, regardless of the time after feeding, with the levels of TG increasing the most. Plasma TG levels at 96 h were nine times higher in E_2-treated animals than in controls (874 mg/dL *vs.* 96 mg/dL). The other parameters assayed, FC, PL, FFA and protein, also increased in treated *vs.* control trout, reaching maxima at 154 h. Starvation in controls was accompanied by a significant decrease in the plasma concentrations of all lipids, except FC (total lipids = 1212 mg/dL at 24 h *vs.* 734 mg/dL at 154 h). In E_2-treated animals rendered hyperlipidemic and hyperproteinemic, only the plasma concentrations of CE and TG decreased during starvation (significant at $P < 0.05$ for TG), while those of PL and proteins increased significantly (PL = 802 mg/dL at 24 h *vs.* 1034 mg/dL at 154 h; proteins = 8019 mg/dL at 24 h *vs.* 11,879 mg/dL at 154 h). During fasting, triglyceridemia

TABLE 1

Changes in the Concentrations of Plasma Lipid Fractions and Proteins at Different Time Points After Feeding with and Without 17β-Estradiol Treatment in Juvenile Trout (mg/dL plasma)[a]

Time after feeding		Total cholesterol (mg/dL)	Free cholesterol (mg/dL)	Cholesteryl ester (mg/dL)	Triglyceride (mg/dL)	Phospholipid (mg/dL)	Free fatty acid (mEq/L)	Total lipids (mg/dL)	Protein (mg/dL)
+24 h	Control (9)	200 ± 27	75 ± 13	211 ± 25	358 ± 89	557 ± 66	0.335 ± 0.060	1212 ± 193	4840 ± 282
	Estradiol (9)	390 ± 50	235 ± 34[c]	262 ± 32	968 ± 28[d]	802 ± 55	0.526 ± 0.044	2323 ± 125[c]	8019 ± 410[d]
+96 h	Control (9)	179 ± 22	64 ± 8	194 ± 24	96 ± 17	437 ± 49	0.224 ± 0.044	799 ± 97	4750 ± 319
	Estradiol (8)	385 ± 43[b]	249 ± 30[d]	230 ± 31	874 ± 36[d]	922 ± 56[d]	0.580 ± 0.093	2291 ± 133[d]	9726 ± 440[d]
+154 h	Control (6)	164 ± 28	61 ± 9	175 ± 35	80 ± 20	416 ± 82	0.115 ± 0.017	734 ± 142	4582 ± 255
	Estradiol (4)	375 ± 45[b]	263 ± 28[d]	191 ± 31	675 ± 79[d]	1034 ± 51[d]	0.516 ± 0.170[b]	2178 ± 133[d]	11879 ± 269[d]

[a]Values are means ± SEM. Sample numbers are given in parentheses. At each time point after feeding, the significances for the differences between control and treated trout were tested using analysis of variance and Scheffe's multiple range test.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

in control trout dropped faster than in E_2-treated trout, but both were decreasing, thus amplifying the difference.

The increase in total plasma cholesterol in E_2-treated trout was not reflected in a concomitant increase in the plasma CE concentration. This indicates that the percentage of esterified cholesterol was lower in these animals and that the increase in cholesterolemia was due only to an increase in free cholesterol. The percentage of esterified cholesterol decreased even during starvation, from 63% in controls to only 30% after 154 h of starvation in E_2-treated animals.

Effects of starvation and 17β-estradiol on lipoprotein profile. The observed changes in plasma concentrations of the different lipids and proteins after feeding and E_2 administration revealed changes in plasma lipoprotein concentrations. A complete lipoprotein profile was obtained by fractionating 10 μL of plasma by density gradient ultracentrifugation. The lipoprotein profile was recorded by continuously monitoring absorbance at 280 nm as lipoproteins were pumped from the centrifuge tube. The different classes of lipoproteins were clearly identifiable (Fig. 2) and have been previously characterized (see ref. 38). Successive blood samples from the same animal, E_2-treated or control, illustrated the changes at different times after feeding.

The peak in alimentary absorption in controls was reflected in a substantial increase in the concentration of the TG-rich lipoproteins, VLDL and chylomicrons (d < 1.015 g/mL), reaching a maximum 16 h after feeding. At the time of the absorption peak and under standardized alimentary conditions, postprandial plasma triglyceridemia and the plasma concentration of the TG-rich lipo-

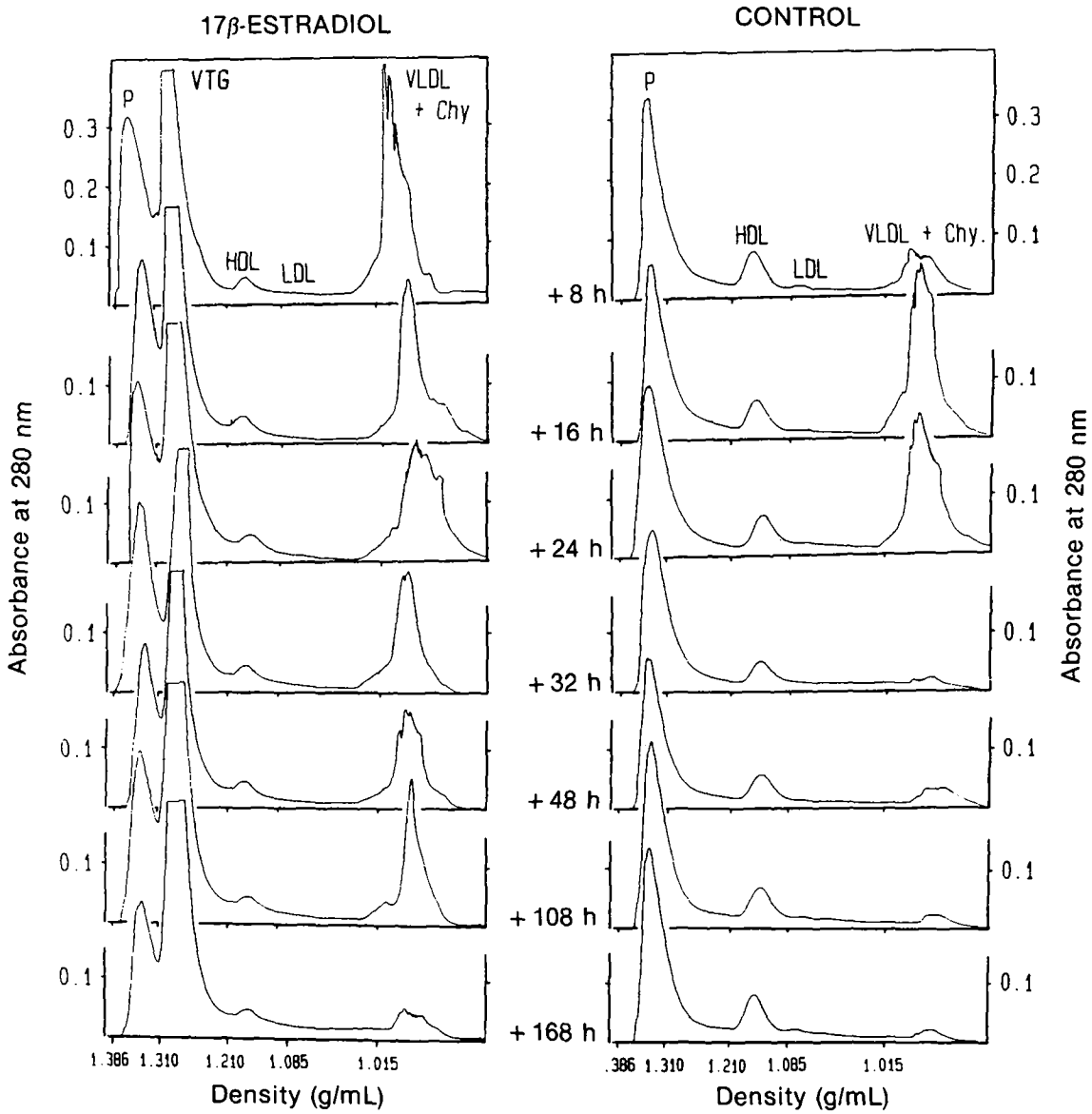

FIG. 2. Effects of starvation and 17β-estradiol on plasma lipoprotein profile in juvenile trout. Ultracentrifugal single-spin absorbance profiles of 10 μL of plasma were obtained at different hours after feeding. The two trout were selected for comparable postprandial triglyceridemia, 1126 mg/dL for the estrogen-treated trout *vs.* 1007 mg/dL for the control trout, 16 h after feeding. HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL + Chy., very low density lipoproteins + chylomicrons; VTG, vitellogenin.

proteins, VLDL and chylomicrons, depended on the quantity of food ingested during the meal (data not shown). To eliminate variations arising from nutritional variables, an E_2-treated trout and a control trout having comparable postprandial triglyceride levels and quantities of lipoproteins of $d < 1.015$ g/mL at the absorption peak were compared (Fig. 2) (TG 1126 *vs.* 1007 mg/dL at 16 h and 1108 *vs.* 957 mg/dL at 24 h; the lipoproteins were quantitated by integrating peak areas).

The administration of E_2 caused a very high concentration of VTG ($1.21 < d < 1.31$ g/mL) in the plasma. The concentrations of VLDL-chylomicrons and VLDL were much higher in E_2-treated trout than in controls. At 32 h, the quantity of circulating VLDL had returned to normal levels in controls (TG = 185 mg/dL), and it remained very high in E_2-treated trout (TG = 999 mg/dL), and lasted up to at least seven days of starvation (TG = 566 mg/dL in E_2-treated *vs.* 156 mg/dL in controls on day 7). The plasma concentration of VLDL-chylomicrons in E_2-treated trout was higher at 8 h than at the time when alimentary absorption peaked (16 and 24 h). The animals had been starved for 72 h (days 7 to 10) before the last feeding (see Fig. 1); therefore the peak at 8 h for the last feeding also represented the VLDL peak at 80 h (72 h + 8 h) for the penultimate feeding. The magnitudes of the VLDL peaks at 48 h and 108 h in Figure 2 clearly indicate that the peak at 8 h resulted from the addition of VLDL from the two feedings.

Concentrations and compositions of plasma lipoproteins. The quantitative changes in the different classes of lipoproteins in both groups of trout are listed in Table 2. Plasma hypertriglyceridemia induced by E_2 was due primarily to a significant increase in the concentration of VLDL, whether at the alimentary absorption peak (VLDL-chylo.: 6 × control level at 24 h) or in starving animals (VLDL: 15 × control level at 96 h). LDL (IDL + LDL), most of which are derived from lipolysis of VLDL, also were present in higher concentrations in E_2-treated animals than in controls, especially during starvation. Thus, 96 h after feeding, the mean concentration of LDL in treated animals was twice that in the controls (492 *vs.* 222 mg/dL), but the difference was not significant. E_2 treatment did not significantly change the concentrations of HDL and plasma proteins in the pellet ($d > 1.31$ g/mL). E_2 induced hepatic synthesis of VTG, a phospholipid-rich lipoprotein (18% lipids, of which 2/3 are phospholipids) which is the major protein in the plasma of E_2-treated trout. Due to the high density and the high concentration of VTG, it was not possible to satisfactorily separate VTG from the plasma proteins in the pellet (see Figs. 2 and 3). The values in Table 2 for juvenile control trout show the protein concentration in the density zone of 1.21 to 1.31 g/mL and do not imply the existence of VTG.

During starvation, the concentration of VLDL decreased in both control and E_2-treated trout, while LDL decreased in the controls (Table 2). The HDL concentration was not significantly changed in either controls or treated animals. The VTG concentration, however, increased significantly (twofold increase between 24 and 154 h), explaining the previously mentioned increase in plasma PL and proteins during starvation in E_2-treated trout (Table 1). The lipoprotein profiles presented in Figure 3 were typical of the mean concentrations induced (Table 2) after E_2 administration and/or starvation; they illustrate the increase in VLDL concentrations after E_2 administration (3492 *vs.* 973 mg/dL at 24 h, 1861 *vs.* 95 mg/dL at 96 h, 1046 mg/dL at 154 h). The progressive increase in VTG concentration during starvation also is well illustrated (3874 mg/dL at 24 h, 4772 mg/dL at 96 h and 8168 mg/dL at 154 h).

The percentages of lipid and protein in VLDL ($d < 1.015$ g/mL) of controls were different at 24 h and 96 h (TG/CE, 50:10, for core lipids and FC/PL/proteins, 5:23:12 for surface components at 24 h; *vs.* 45:14 and 6:23:12 at 96 h; in weight ratios). At 96 h, the core lipids were depleted in TG and enriched in CE. By contrast, the compositions of E_2-treated VLDL were identical at 24 h and 96 h (TG/CE, 49:6 for core lipids and FC/PL/proteins, 6:20:19, for surface components). However, the compositions of VLDL of E_2-treated trout and control trout were different. CE was depleted in E_2-treated VLDL, especially at 96 h, whereas protein was enriched. The increase in total plasma cholesterol in E_2-treated trout was not reflected in a concomitant increase in plasma CE concentration (Table 1). This was consistent with the high concentration of VLDL depleted in CE in E_2-treated trout.

A comparison of the VLDL apolipoprotein profiles of

TABLE 2

Effects of 17β-Estradiol Administration and Fasting on Plasma Lipoprotein Concentrations in Juvenile Trout (mg/dL plasma)[a]

Time after feeding		Bottom proteins (P)	Vitellogenin (VTG)	High density lipoproteins (HDL)	Low density lipoproteins (LDL)	Very low density lipoproteins (VLDL)
+24 h	Control	2405 ± 372(4)	1274 ± 233(4)	852 ± 172(5)	395 ± 122(5)	307 ± 158(5)
	Estradiol	1874 ± 123(7)	3836 ± 283(7)[b]	1117 ± 138(6)	547 ± 64(6)	2716 ± 50(7)[b]
+96 h	Control	2122 ± 352(4)	1052 ± 206(4)	838 ± 145(4)	222 ± 76(4)	118 ± 34(4)
	Estradiol	1494 ± 108(7)	5314 ± 368(7)[c]	1112 ± 108(7)	492 ± 32(7)	1861 ± 391(7)[b]
+154 h	Control	2017 ± 160(3)	901 ± 187(3)	657 ± 132(3)	73 (2)	189 (2)
	Estradiol	2372 ± 626(3)	6971 ± 976(3)[c]	1659 (2)	684 ± 131(3)	1179 ± 254(3)

[a]Values are means ± SEM. Sample numbers are given in parentheses. A density gradient ultracentrifugation procedure was used for the fractionation of whole plasma lipoproteins and proteins. The concentrations were calculated as follows: total lipoproteins = cholesteryl esters + free cholesterol + triglycerides + phospholipids + proteins. Density regions used were VLDL (VLDL + chylomicrons at + 24 h after feeding), $d < 1.015$ g/mL; LDL (LDL + IDL), $1.015 < d < 1.085$ g/mL; HDL, $1.085 < d < 1.210$ g/mL; VTG (presence of VTG only for estrogen-treated fish), $1.210 < d < 1.310$ g/mL; P (bottom proteins), $d > 1.310$ g/mL. At each time point after feeding, the significances of the differences between control and treated trout were tested using analysis of variance and Scheffe's multiple range test.

[b]$P < 0.05$.

[c]$P < 0.001$.

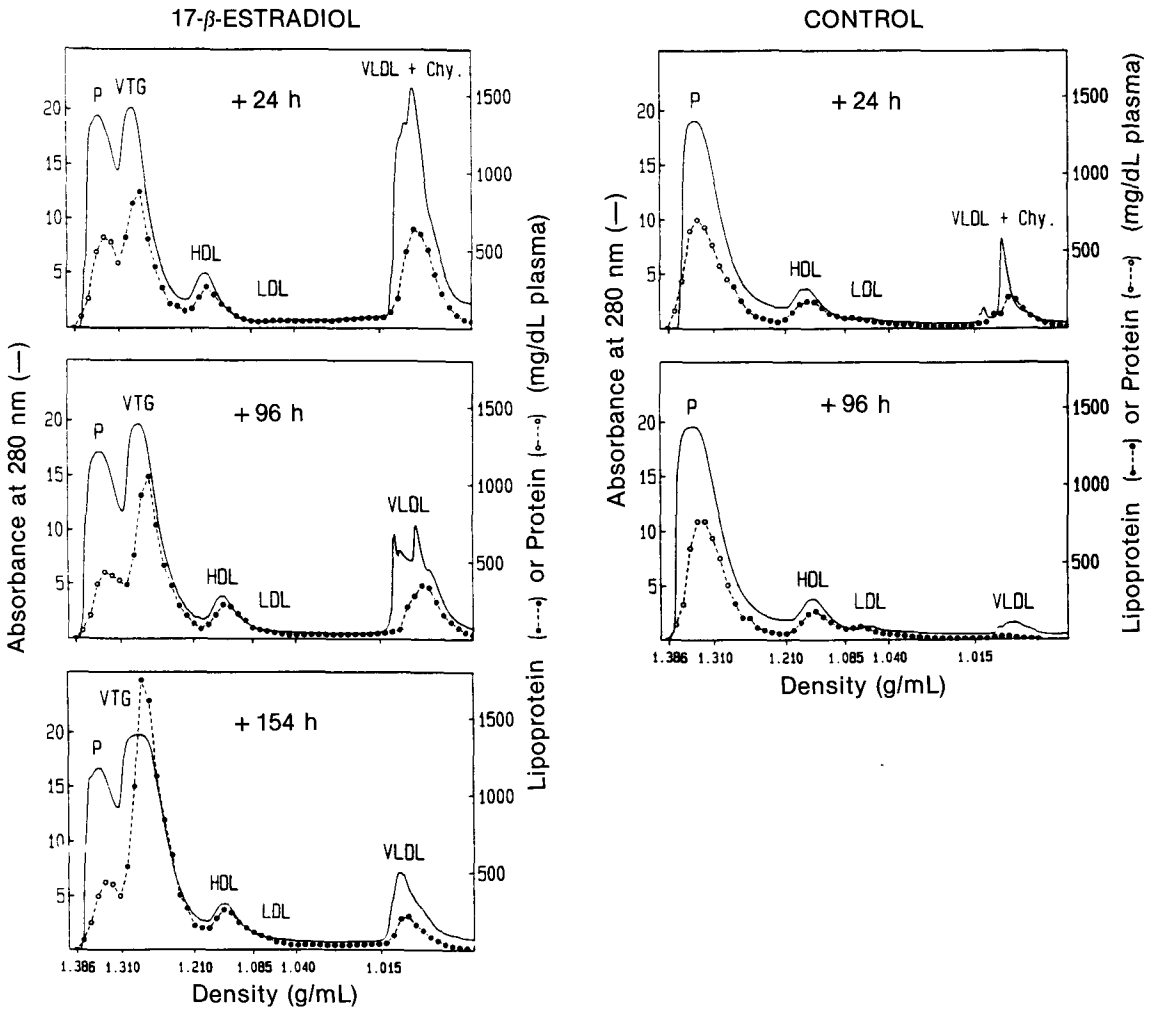

FIG. 3. Density gradient ultracentrifugal lipoprotein quantitative profiles of a 17β-estradiol (E_2)-treated or a control juvenile trout obtained at different times after feeding. The lipoprotein profiles presented were typical of the mean concentrations induced (Table 2) after E_2 administration and/or starvation. Absorbance at 280 nm (——), lipoprotein (when lipids were present in the fraction) (●—●—●), or protein (O—O—O) profiles. The lipoprotein concentration was calculated from the expression: total lipoproteins = cholesteryl esters + free cholesterol + triglycerides + phospholipids + proteins. The concentrations are expressed in mg/dL plasma. Abbreviations as in Figure 2.

control and E_2-treated trout did not show any new apolipoproteins in the E_2-treated animals (Fig. 4). At 24 h, the electrophoretic profiles were similar, but marked changes in the distribution of apolipoproteins were observed at 154 h. In control trout, prolonged starvation resulted in the total disappearance of Mr 240,000 apolipoprotein (apo B_{240}) which persisted in VLDL of E_2-treated trout. In these VLDL there was also a depletion of Mr 76,000 and 66,000 apolipoproteins at 154 h and an enrichment in species of Mr 25,000 (apo A-I) and Mr 13,000 (apo A-II).

The plasma concentration of VLDL in estrogenized trout was very high 96 h after feeding (Fig. 3). Electron microscopy of these VLDL (Fig. 5B) indicated that their size was homogeneous and that their mean diameter was lower than at the peak of alimentary absorption (24 nm *vs.* 20–50 nm) (31,38).

Ultrastructural study. The ultrastructural analysis of liver cells from estrogenized trout 96 h after feeding showed active synthesis of small VLDL, with a diameter similar to that observed in plasma (Fig. 5A and 5B). The cavities of the endoplasmic reticulum contained particles the size of VLDL which accumulated on the Golgi complex formation face. The extremities of the Golgi complexes formed secretion grains which released lipoproteins into intercellular space. The same feature was observed for control trout; however, at the end of the experiment, the LSI of E_2-treated and control animals were significantly different ($P < 0.001$): 4.01 ± 0.25% (n = 8) *vs.* 1.35 ± 0.11% (n = 9) and indicated hepatomegaly in the treated animals.

Triglyceride and VLDL-triglyceride output in vivo. The levels of plasma TG secreted by E_2-treated and control trout were determined *in vivo* after 96 h of starvation, after injecting Triton WR-1339.

Mean TG increases were 45.2 ± 14.6 mg/dL (n = 7) in E_2-treated animals *vs.* 15.1 ± 2.8 mg/dL (n = 8) in controls 3 h after injecting Triton, and 78.5 ± 34 mg/dL

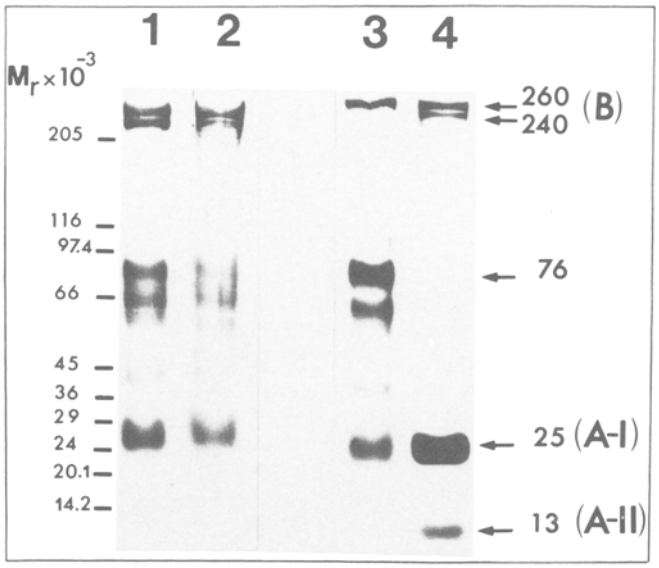

FIG. 4. Electrophoretic patterns in sodium dodecylsulfate/glycerol/polyacrylamide gradient gel of very low density lipoprotein (VLDL)-apolipoproteins from control and 17β-estradiol-treated trout (75 μg of proteins for lanes 1 and 2, 50 μg of proteins for lanes 3 and 4). 1, control VLDL 24 h after feeding; 2, 17β-estradiol-treated VLDL 24 h after feeding; 3, control VLDL 154 h after feeding; 4, 17β-estradiol-treated VLDL 154 h after feeding. Mr × 10^{-3}, reference markers relative molecular weight × 10^{-3}; (B), apo B; (A-I) apo A-I; (A-II), apo A-II (see references 31, 38 and 39 for details about trout apolipoproteins).

(n = 8) *vs.* 17.1 ± 3.2 mg/dL (n = 8) 6 h after injecting Triton. Based on these values the mean TGSR could be calculated as: 0.086 mg/h in controls and 0.308 mg/h in treated animals (control × 3.5) for a mean body of 63 g. The LSI values at the end of the experiment were significantly different ($P < 0.001$): 2.786 ± 0.23% (n = 11) in treated animals *vs.* 1.40 ± 0.06% (n = 11) in controls.

The difference in VLDL output, during starvation, between E_2-treated and control trout was determined by reproducibly isolating a part of plasma VLDL by microultracentrifugation 3 h after injecting Triton. Pure VLDL, not contaminated by other lipoprotein classes, were isolated. However, the volume collected was too small to completely recover the VLDL fraction. Under these conditions, the mean increases in VLDL-TG concentrations were significantly different: 17.1 ± 7.8 mg/dL (n = 5) in treated animals *vs.* 1.2 ± 0.4 mg/dL (n = 6) in controls.

DISCUSSION

Fish are poikilothermic vertebrates which preferentially utilize lipids rather than carbohydrates as energy source (59,60). Ovarian growth and reproduction activity are reflected by a clearcut increase in energy demand (61). There are two sources for this energy, one exogenous and of alimentary origin, the other endogenous, from lipid storage tissues (muscle, liver and possibly perivisceral adipose tissue) which are characterized by a succession of phases of deposition and mobilization of lipid reserves. Exogenous vitellogenesis, *i.e.*, endocytosis of the yolk precursors by oocytes, is shown by, among other things, elevated plasma concentrations of E_2 and testosterone, hypertrophy of the liver and the ovaries, and profound changes in lipid metabolism. These are reflected in qualitative and quantitative changes in the different classes of plasma lipoproteins.

Trout exhibit seasonal variations, which still are poorly defined, in the levels of the different classes of plasma lipoproteins other than VTG, and which are related to the annual reproductive cycle (31,62-64). In particular, the VLDL concentration increases during exogenous vitellogenesis (62,64). Studies on the effects of sex steroids on plasma lipids unrelated to VTG have been few in fish (65,66). No data exist on the possible role of sex hormones in the metabolic regulation of various classes of plasma lipoproteins other than VTG.

The injection of E_2 into juvenile trout enabled us to obtain LSI and VTG concentrations comparable to those in adult trout in exogenous vitellogenesis (31,67). Our results show that in addition to inducing VTG synthesis, E_2 led to a considerable increase in the concentration and secretion of VLDL in plasma. As in chicks (68) and rats (18), the increased concentration of VLDL appears to be due to stimulated hepatic secretion of VLDL. These effects on VLDL are apparently common to all vertebrates (11,14,17,19), particularly oviparous (24-26,68). Induced hypertriglyceridemia in trout arises primarily from the increased concentration of the triglyceride-rich VLDL and, to a lesser extent, LDL, whereas VTG in this species contains only 4% TG (69). In fish, as in mammals, most LDL are derived from the sequential lipolysis of VLDL to IDL and then LDL (38,70), which would explain the slightly increased concentration of LDL in E_2-treated trout.

Hyperlipidemia and hyperproteinemia induced by E_2 in trout are at least partially due to increased secretion of lipoproteins, which persists after several days of starvation, consistent with recent observations in chicks (71). The increased levels of FFA under the effect of estrogens, which are especially pronounced during starvation in trout (as shown here), but also in chicks (71,72) and lizards (73), suggest that estrogens also may cause the mobilization of fatty acids in reserve tissues (71,73-75).

Alimentary assimilation is slower in fish than in mammals (31). The appearance of TG-rich lipoproteins in plasma, characteristic of the postprandial absorption peak, thus occurs later in trout than in humans. After the postprandial phase, progressive decreases in VLDL and LDL concentrations were observed, while HDL levels stayed the same, in agreement with other observations in trout during starvation (76). The very high concentrations of VLDL in E_2-treated animals also were associated with a slow and gradual decrease in VLDL concentrations during starvation, indicating that plasma clearance is effective and/or hepatic secretion is reduced. The progressive increase in VTG concentration during starvation may be due to VTG accumulation in plasma of juvenile trout, in the absence of ovarian uptake.

In chicks, the VLDL present in plasma during E_2 treatment are very different from the VLDL of controls, especially in size, electrophoretic migration and apolipoprotein composition (77). The VLDL of E_2-treated chicks are composed of at least two different lipoprotein classes with different particle sizes and apolipoprotein compositions. Small VLDL were predominant in E_2-treated chicks (24,77).

In fish, as in other vertebrates, the peak of alimentary absorption results in a mixture of TG-rich lipoproteins of

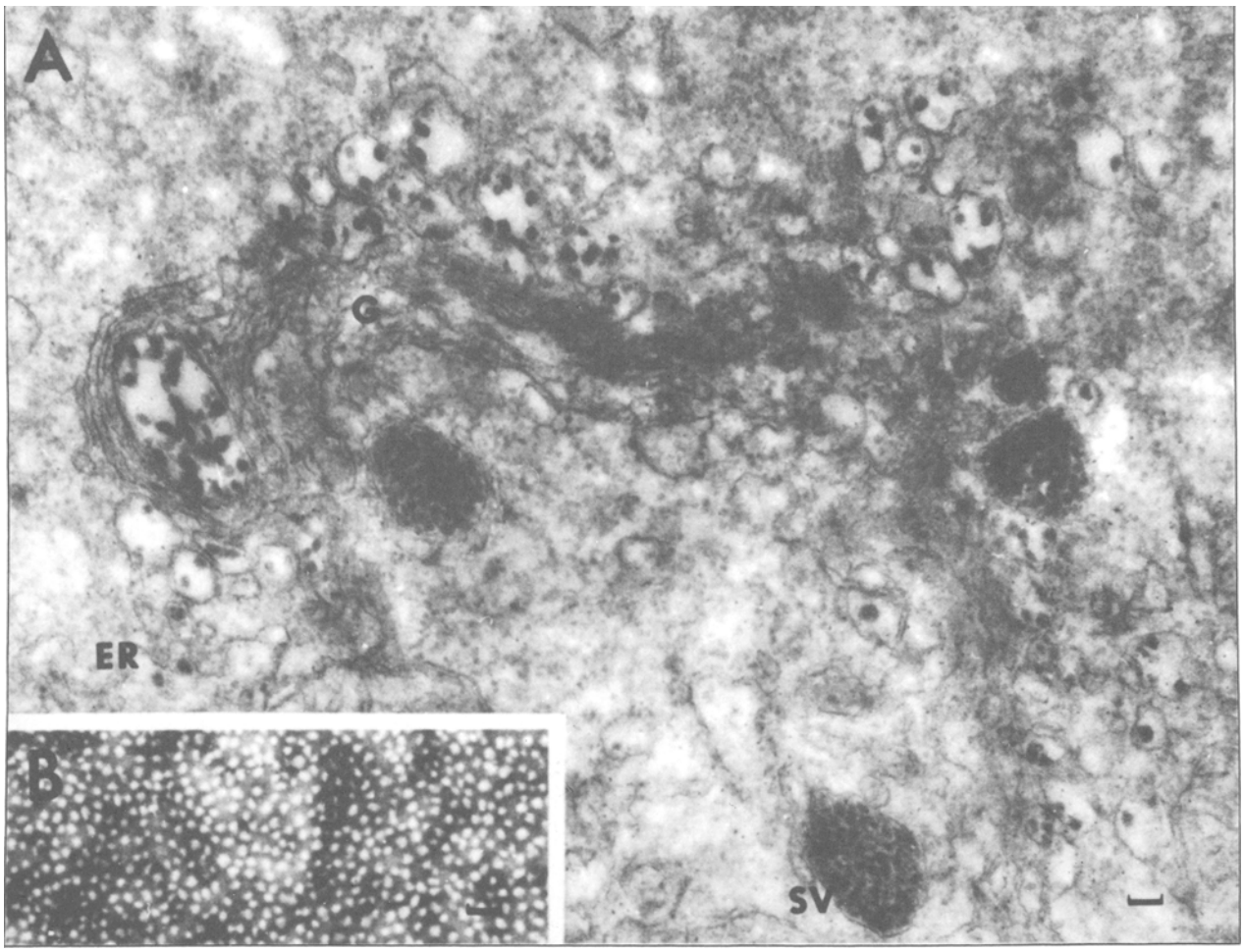

FIG. 5. Liver cell ultrastructure (A) and plasma VLDL (B) of juvenile estrogenized trout in the course of fasting (96 h after feeding). (A), ultrastructural analysis was carried out on sections in which lipids were contrasted by the OTO method. Very low density lipoproteins appeared as black particles in endoplasmic reticulum (ER), Golgi complex (G), and secretory vesicles (SV). (B), lipoproteins were isolated by density gradient ultracentrifugation (d < 1.015 g/mL), positively stained with OsO_4, and then negatively stained with phosphotungstate. Scale bar = 100 nm.

intestinal (78,79) and hepatic origin (79) in the plasma. Fasting results in a progressive disappearance of TG-rich intestinal lipoproteins (78). In E_2-treated animals, there is a high concentration of small VLDL in the plasma, similar to those synthesized by the liver, and being enriched in certain apolipoproteins. These results suggest that treatment with E_2 may change the apolipoprotein distribution of endogenous hepatic VLDL.

The increased rate of secretion of TG or VLDL-TG after injecting Triton WR-1339 in fasting animals, between control and estrogenized trout, may be partially due to the increase in the LSI. This index, increased by a factor of two or three in estrogenized trout, indicates hepatomegaly in these animals. Hepatomegaly is coupled with active synthesis of VLDL by liver cells and may be responsible for the very high concentration of plasma VLDL in estrogenized fish. The increase in net secretion of hepatic VLDL does not exclude the possibility of a simultaneous decrease in the activities of plasma lipases or in the production of VLDL particles, which are less sensitive to the action of LPL. Aside from the modified CE and protein contents of the entire VLDL pool and an enrichment of the fraction in certain apolipoproteins during starvation, we were unable to show qualitative differences in the apolipoprotein composition of VLDL between E_2-treated and control trout (38,39). In chicks, the presence of apo VLDL-II in VLDL after E_2 treatment is reflected in a decrease in their sensitivity to LPL (27,28) and by slower catabolism of the particles in the plasma of males (77). In laying hens, this decreased sensitivity toward LPL enables these VLDL to be incorporated in growing oocytes (80), mediated by an apo B-specific surface receptor (29) which is identical to the VTG receptor (30). Even though no direct proof exists, the strategy appears to be different in trout. In addition to taking up VTG (81) by a specific surface receptor (82), the ovary may be able to take up lipid components by lipolysis of circulating lipoproteins. This is suggested by the considerable increase in LPL and salt-resistant lipase activities in the ovary during exogenous vitellogenesis (63). LPL in this species is greatly activated by VLDL and, to a lesser extent, by HDL (83).

In conclusion, estrogen-induced hyperlipidemia in the juvenile trout results in major changes in lipoprotein profiles. Estrogen-induced changes in the profiles can occur spontaneously in females during the reproductive cycle. The increased concentration of VLDL and the appearance

of VTG after administering E_2 are similar to those observed in chicks and suggest the existence of similar mechanisms for the regulation of lipoprotein metabolism by estrogens in oviparous vertebrates.

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METHOD

A Method for Determination of the Absolute Stereochemistry of α,β-Epoxy Alcohols Derived from Fatty Acid Hydroperoxides

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A method for the determination of the absolute configuration of the alcohol group of fatty acid α,β-epoxy alcohols was developed. The method consists of: (i) deoxygenation of the saturated epoxy alcohol to an allylic alcohol by treatment with triphenylphosphine selenide and trifluoroacetic acid; (ii) oxidative ozonolysis of the (−)-menthoxycarbonyl derivative of the allylic alcohol; and (iii) steric analysis of the resulting 2-hydroxy acid (methyl ester, (−)-menthoxycarbonyl derivative) by gas-liquid chromatography using appropriate reference compounds. The result obtained, coupled with knowledge of the relative configuration of the epoxy alcohol *(erythro/threo)* and of the geometrical configuration of the epoxide group *(cis/trans)*, permitted assignment of the absolute configuration of all three asymmetric carbons of the α,β-epoxy alcohol. The method was applied to the determination of the absolute stereochemistry of two hepoxilins recently isolated from the red alga *Murrayella periclados*. *Lipids 27,* 1042-1046 (1992).

A variety of regio- and stereoisomeric fatty acid epoxy alcohols are formed from unsaturated fatty acid hydroperoxides. Epoxy alcohols of types **I** and/or **II** (Fig. 1) have been obtained in studies where hydroperoxides have been exposed to heat (1), Fe^{2+} (2), hemoglobin (3), hematin (4), soybean lipoxygenase (5), acid (6) and rat lung homogenate (7). Formation of these types of epoxy alcohols involves homolytic or heterolytic cleavage of the O–O bond of the parent hydroperoxide, followed by attack by the proximal hydroperoxide oxygen at the α-unsaturation to produce the epoxide function. The epoxyallylic radical or carbocation is subsequently attacked by either dioxygen, the distal hydroperoxide oxygen or solvent, to provide a non-allylic α,β-epoxy alcohol (type **I**) or an allylic γ,δ-epoxy alcohol (type **II**) (8). Epoxy alcohols of types **III** and **IV** have been obtained by enzymic reactions, *i.e.,* following incubation of hydroperoxides with a fatty acid hydroperoxide isomerase from the fungus *Saprolegnia parasitica* (9) and with a hydroperoxide-dependent epoxygenase from *Vicia faba* (10,11). In addition, epoxy alcohols of type **III** are produced from hydroperoxides by intermolecular epoxidation catalyzed by vanadium oxyacetylacetonate (12).

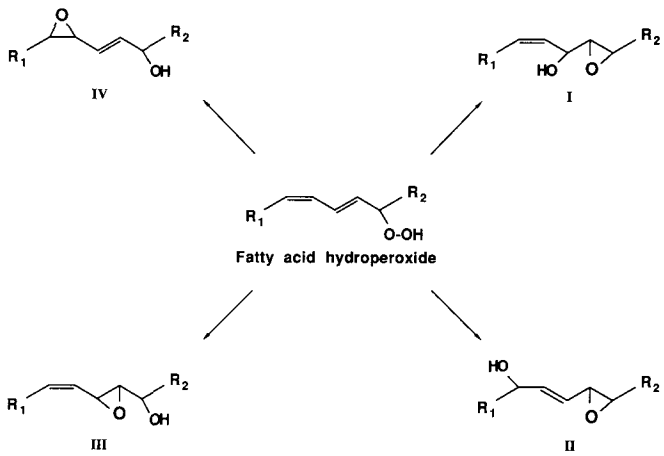

FIG. 1. Rearrangement of fatty acid hydroperoxides into epoxy alcohols.

The configuration of the allylic alcohol group of γ,δ-epoxy alcohols (types **II** and **IV** in Fig. 1) has been determined by cleavage of the double bond followed by steric analysis of the resulting 2-hydroxy acid (see *e.g.,* refs. 9 and 13). In addition, these epoxy alcohols, as well as epoxy alcohols of type **III**, are easily hydrolyzed to derivatives containing a 1,2,5-trihydroxy-3*E*-pentene structure. Methodology for determination of the absolute stereochemistry of such trihydroxy fatty acids has been developed (14) and can be utilized for assignment of the stereochemistry of the parent allylic epoxy alcohol. On the other hand, in the case of epoxy alcohols of type **I**, neither chemical degradation nor acid-catalyzed hydrolysis into trihydroxy derivatives has been useful for determining the absolute configuration. The present paper describes a simple method to establish the absolute configuration of the allylic hydroxyl group of this type of epoxy alcohols. The method, in combination with existing chemical and physical methods for determining relative configuration, allows assignment of the absolute stereochemistry of epoxy alcohols of type **I**.

MATERIALS AND METHODS

Linoleic acid was purchased from Nu-Chek Prep (Elysian, MN), and [1-^{14}C]linoleic acid was obtained from Amersham Laboratories (Amersham, U.K.). [1-^{14}C]-Labeled 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*S*-[1-^{14}C]HPOD; specific radioactivity, 2.1 kBq/μmol; enantiomeric composition, 13*S*/13*R*, 98:2) was obtained by incubation of [1-^{14}C]linoleic acid with soybean lipoxygenase (1). Triphenylphosphine selenide was purchased from Fluka (Buchs, Switzerland), and human hemoglobin was obtained from Sigma Chemical Co. (St. Louis, MO).

Methyl erythro-*11-hydroxy*-trans-*12,13-epoxy-9Z-octadecenoate.* The methyl esters of the *erythro* and *threo* isomers of the α,β-epoxy alcohol 11-hydroxy-12,13-epoxy-9-octadecenoic acid were prepared by modification of a procedure described earlier (3). Thus, 13(*S*)-[1-^{14}C]HPOD (32 mg) was added to a solution of 2 g of hemoglobin in 20 mL of potassium phosphate buffer, pH 7.4, and the mixture was shaken at 37°C for 10 min. In order to remove

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Abbreviations: FTIR, Fourier-transform infrared; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; hepoxilin B_3, 10-hydroxy-11,12-epoxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid; hepoxilin B_4, 10-hydroxy-11,12-epoxy-5*Z*,8*Z*,14*Z*,17*Z*-eicosatetraenoic acid, 13*S*-HOD, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid; 13*S*-HPOD, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; HPLC, high-performance liquid chromatography; MC, (−)-menthoxycarbonyl; Me_3Si, trimethylsilyl; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; UV, ultraviolet.

the γ,δ-epoxy alcohol 9*R,S*-hydroxy-12,13-epoxy-10-octadecenoic acid (3), which interfered with the isolation of the more polar of the two α,β-epoxy alcohols, the reaction mixture was acidified to pH 2 and kept at this pH at 23°C for 3 min. The mixture was extracted with two volumes of diethyl ether, and the combined ether phases were washed until neutral. The residue obtained following evaporation of the solvent was esterified by treatment with diazomethane and subjected to preparative thin-layer chromatography (TLC). Five main products appeared, *i.e.*, methyl 13-oxo-9,11-octadecadienoate (14%, $R_f = 0.74$), the methyl ester of 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HOD, 32%, $R_f = 0.55$), methyl *erythro*-11-hydroxy-12,13-epoxy-9-octadecenoate (7%, $R_f = 0.40$), material (10%, $R_f = 0.35$) mainly due to methyl *threo*-11-hydroxy-12,13-epoxy-9-octadecenoate, as well as polar material (26%, $R_f = 0$–0.02) mainly due to stereoisomers of methyl 9,10,13- and 9,12,13-trihydroxyoctadecenoates formed from 9*R,S*-hydroxy-12,13-epoxy-10-octadecenoic acid in the acid treatment step.

Analysis of the trimethylsilyl (Me_3Si) derivative of methyl *erythro*-11-hydroxy-12,13-epoxy-9-octadecenoate by gas chromatography/mass spectrometry (GC/MS) showed a C value (15) of 20.62. The mass spectrum showed a prominent ion at *m/e* 285 (Me_3SiO^+=CH-CH=CH-$(CH_2)_7$-$COOCH_3$; 100%) as well as ions at *m/e* 398 (M, 0.5), 383 [M − 15 (loss of $\cdot CH_3$), 1], and 327 [M − 71 (loss of $\cdot C_5H_{11}$), 3]. That the relative configuration at C-11/C-12 was *erythro* followed from previous work which had shown that $LiAlH_4$ reduction of the saturated epoxy alcohol resulted in the formation of similar amounts of a 11,13-dihydroxy-1-octadecanol and *erythro*-11,12-dihydroxy-1-octadecanol (3). Infrared spectrometric analysis of methyl *erythro*-11-hydroxy-12,13-epoxy-9-octadecenoate showed bands at *inter alia* 3620–3350 cm^{-1} (hydroxyl), 1730 cm^{-1} (ester carbonyl) and 890 cm^{-1} (*trans*-epoxide). No absorption band appeared in the region 950–1000 cm^{-1}, thus showing that the configuration of the Δ9 double bond remained *Z*. These experiments confirmed and extended the previous structural work (3) and showed that the epoxy alcohol was identical with methyl *erythro*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoate.

Methyl threo-*11-hydroxy*-trans-*12,13-epoxy-9Z-octadecenoate.* 13*S*-[1-^{14}C]HPOD was treated with hemoglobin as described above, and the esterified product was subjected to TLC. Material present in the band at $R_f = 0.35$ was previously found to be due to *ca.* 80% methyl *threo*-11-hydroxy-12,13-epoxy-9-octadecenoate and 20% of an *erythro* isomer, probably methyl *erythro*-11-hydroxy-*cis*-12,13-epoxy-9-octadecenoate (3). The material was subjected to reversed-phase high-performance liquid chromatography (HPLC) affording pure methyl *threo*-11-hydroxy-12,13-epoxy-9-octadecenoate (effluent volume, 23.4–26.1 mL). The C value of the Me_3Si derivative of methyl *threo*-11-hydroxy-12,13-epoxy-9-octadecenoate was 20.64, and the mass spectrum was similar to that recorded on the Me_3Si derivative of the corresponding *erythro* isomer. Steric analysis of 11,12-dihydroxy-1-octadecanol obtained following reduction of the saturated epoxy alcohol with $LiAlH_4$ (3) confirmed the *threo* relationship at C-11/C-12. The infrared spectrum of the epoxy alcohol showed bands at 3620–3350 cm^{-1} (hydroxyl), 1730 cm^{-1} (ester carbonyl) and 890 cm^{-1} (*trans*-epoxide), but no band in the region 950–1000 cm^{-1}. These data demonstrated that the epoxy alcohol was identical to methyl *threo*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoate.

Hepoxilins B_3 and B_4. Hepoxilins B_3 and B_4, isolated from the red alga *Murrayella periclados* (*cf.* ref. 16), were kindly supplied by Drs. M. W. Bernart and W. H. Gerwick, College of Pharmacy, Oregon State University, Corvallis, OR. The Fourier-transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectra recorded on the methyl esters demonstrated that the configurations at C-10/C-11 of the samples of hepoxilins B_3 and B_4 were *erythro* and *threo*, respectively, and that the configuration of the 11,12-epoxide group of both hepoxilins was *trans*.

Dimethyl 2S-hydroxydodecane-1,12-dioate. Dimethyl 2*S*-hydroxydodecane-1,12-dioate was prepared by anodic coupling of ethyl 2*S*-acetoxy-3-carboxypropionate (ref. 17; 5 mmol) and methyl hydrogen sebacate (15 mmol) in methanol (20 mL) containing sodium methoxide (0.8 mmol). A current of 0.4 A was passed through the solution for 2 h. Subsequently, the volume of the solution was adjusted to 60 mL by addition of methanol, and the product was transesterified by treatment with sodium methoxide (0.5 M). After 15 min at 23°C, water and hydrochloric acid were added and the solution extracted twice with diethyl ether. The isolated material was subjected to silicic acid column chromatography. Elution with diethyl ether/hexane (1:3, vol/vol) yielded dimethyl 2*S*-hydroxydodecane-1,12-dioate (0.5 g; yield, 36%), which was crystallized from hexane at −30°C. The identity of the material was confirmed by mass spectrometric analysis of the Me_3Si derivative. Prominent ions were observed at *m/e* 331 (M − 15, 2%), 315 [M − 31 (loss of $\cdot OCH_3$), 6], 303(14), 287 [Me_3SiO^+=CH-$(CH_2)_9$-$COOCH_3$, 100], 271(29), and 183 [OHC-$(CH_2)_9$-C≡O^+, 14]. The optical purity of the material was better than 99% as judged by gas-liquid chromatography (GLC) analysis of the (−)-menthoxycarbonyl (MC) derivative (*cf.* ref. 18).

Dimethyl 2R,S-hydroxydodecane-1,12-dioate. Dimethyl 2*S*-hydroxydodecane-1,12-dioate (16 mg) was oxidized with CrO_3 (6 mg) in 97.5% acetic acid (2.4 mL) at 37°C for 30 min. The ketodiester was isolated by preparative TLC (solvent system: ethyl acetate/hexane, 15:85, vol/vol; $R_f = 0.42$). The mass spectrum showed prominent ions at *m/e* 272 (M, 0.5%), 241 (M − 31, 14), 213 [M − 59 (loss of $\cdot COOCH_3$), 100], 181 [M − (59 + 32), 7], 153(28), and 135(33). An aliquot of the dimethyl 2-ketododecane-1,12-dioate was treated with $NaBH_4$ in methanol. GLC analysis of the MC derivative of the resulting dimethyl 2*R,S*-hydroxydodecane-1,12-dioate showed two peaks (areas, 1:1) due to the MC derivatives of the *S* and *R* hydroxydodecanedioates. In accordance with previous results (18,19), the first eluting diastereomer was the MC derivative of dimethyl 2*S*-hydroxydodecanedioate.

Catalytic hydrogenation. The unsaturated compound (0.1–2 mg) was stirred with 20 mg of palladium on calcium carbonate (E. Merck, Darmstadt, Germany) in 3 mL of ethyl acetate under hydrogen gas for 1 h. Use of this catalyst rather than the more active platinum or palladium on carbon catalysts considerably reduced the extent of hydrogenolysis of allylic alcohol groups.

Deoxygenation. The epoxy alcohol (0.1–2 mg) was dissolved in methylene chloride (1 mL) containing trifluoroacetic acid (3.5 mg), and triphenylphosphine selenide (31 mg) was added (20). After 15 h at 23°C the mixture was added to diethyl ether (50 mL) and the solution was

washed with water and taken to dryness. The residue was subjected to TLC.

Steric analysis. The allylic alcohol (0.05–1 mg) was derivatized by treatment with toluene (50 μL), pyridine (10 μL) and a toluene solution (50 μL) of (−)-menthoxycarbonyl chloride (Aldrich-Chemie, Steinheim, Germany; 1 μmol/μL) at 23°C for 30 min (14). The MC derivatives were purified by TLC (solvent system; ethyl acetate/hexane, 7:93, vol/vol) and subjected to oxidative ozonolysis as described (18). Material obtained following ozonolysis was esterified by treatment with diazomethane and subsequently analyzed by GLC. MC derivatives of synthetic 2*S*- and 2*R*,*S*-hydroxy-alkanoates/alkanedioates were used as references.

Chromatographic and instrumental methods. TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck and, unless otherwise indicated, a solvent system composed of ethyl acetate/hexane (25:75, vol/vol). Material was located by spraying with 2′,7′-dichlorofluorescein and viewing under ultraviolet (UV) light. Radioactivity on TLC plates was determined with a Berthold Dünnschichtscanner II (Wildbad, Germany) interfaced with a Macintosh SE/30 PC. Silicic acid chromatography was carried out with glass columns packed with silicic acid (Mallinckrodt, Paris, KY; 100 mesh, activated at 120°C). The columns were eluted under pressure with increasing concentration of diethyl ether in hexane. Reversed-phase HPLC was performed using a column of Nucleosil 5 C_{18} (250 × 4 mm) purchased from Machery-Nagel (Düren, Germany) and acetonitrile/water (6:4, vol/vol) at a flow rate of 1.5 mL/min. The absorbancy of the effluent at 210 nm was monitored. GLC was performed with a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Helium at a flow rate of 25 cm/s was used as the carrier gas. GC/MS was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. Infrared spectra were recorded with a Perkin-Elmer (Überlingen, Germany) model 257 infrared spectrophotometer. Radioactivity was determined with a Packard (Downers Grove, IL) Tri-Carb model 4450 liquid scintillation counter.

RESULTS AND DISCUSSION

The present method for the determination of the stereochemistry of α,β-epoxy alcohols was based on deoxygenation of the epoxide function followed by steric analysis of the resulting allylic alcohol. Deoxygenation was carried out by treatment with triphenylphosphine selenide and trifluoroacetic acid in methylene chloride (Fig. 2). This method for deoxygenation of epoxides was originally described by Clive and Denyer (20) and was found to work satisfactorily with epoxy alcohols in the present study. As expected (20), the reaction proceeded stereospecifically to produce *E*-allylic alcohols from the *trans*-epoxides used in the present work. The yields of allylic alcohols were limited to 30–50%, mainly because of further reaction to produce the corresponding trifluoroacetates. Determination of the absolute configuration of the allylic alcohol was achieved following derivatization into the MC derivative, oxidative ozonolysis and GLC analysis of the resulting 2-hydroxy acid (methyl ester, MC derivative). The method

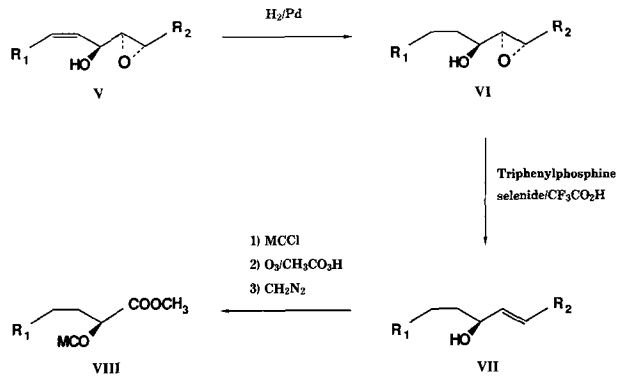

FIG. 2. Reactions used for steric analysis of α,β-epoxy alcohols as applied to methyl *erythro*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoate. V, methyl 11*S*-hydroxy-12*S*,13*S*-epoxy-9*Z*-octadecenoate; VI, methyl 11*S*-hydroxy-12*S*,13*S*-epoxyoctadecanoate; VII, methyl 11*S*-hydroxy-12*E*-octadecenoate; VIII, (−)-menthoxycarbonyl derivative of dimethyl 2*S*-hydroxydodecane-1,12-dioate. R_1, $(CH_2)_7$-$COOCH_3$; R_2, $(CH_2)_4$-CH_3. "MCCl," (−)-menthoxycarbonyl chloride.

was used to confirm the absolute stereochemistry of methyl *erythro*- and *threo*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoates obtained from linoleic acid 13*S*-hydroperoxide in the presence of hemoglobin. In addition, the method was applied to establish the absolute stereochemistry of *erythro*-hepoxilin B_3 and *threo*-hepoxilin B_4 that had been recently isolated as natural products from the red alga *Murrayella periclados*.

Absolute stereochemistry of methyl erythro-*11-hydroxy-trans-12,13-epoxy-9Z-octadecenoate.* A sample of methyl *erythro*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoate (1 mg; V in Fig. 2) was hydrogenated and an aliquot of the product was converted into the Me_3Si derivative and analyzed by GC/MS. The C value recorded was 21.08 and the mass spectrum showed a prominent ion at *m/e* 287 [Me_3SiO^+=CH-$(CH_2)_9$-$COOCH_3$, 64%], as well as weaker ions at *m/e* 385 (M − 15, 11), 301(8), and 215 [M − 185 (loss of ·$(CH_2)_9$-$COOCH_3$), 12]. The hydrogenated epoxy alcohol (**VI**, Fig. 2) was treated with triphenylphosphine selenide and trifluoroacetic acid and the product was subjected to TLC. Two major compounds appeared, *i.e.*, methyl 11-hydroxy-12-octadecenoate (R_f = 0.51, 41%) and a nonpolar by-product (R_f = 0.83, 29%), tentatively identified as the trifluoroacetate of methyl 11-hydroxy-12-octadecenoate. The C value of the Me_3Si derivative of the methyl 11-hydroxy-12-octadecenoate was 19.77, and the mass spectrum showed a prominent ion at *m/e* 199 (Me_3SiO^+=CH-CH=CH-C_5H_{11}, 100%), as well as weaker ions at 384 (M, 1), 369 (M − 15, 2), 337 [M − (15 + 32), 5] and 313 (M − 71, 3). The infrared spectrum of the allylic alcohol showed an absorption band at 970 cm^{-1}, demonstrating that the double bond had the *E* configuration. Oxidative ozonolysis performed on the MC derivative (see below) localized the double bond to the Δ12 position. Thus, the allylic alcohol formed by deoxygenation of the hydrogenated derivative of methyl *erythro*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoate was identical to methyl 11-hydroxy-12*E*-octadecenoate (**VII**, Fig. 2).

Figure 3 (C and D) shows a gas chromatographic analysis of the esterified product obtained following oxidative ozonolysis of the MC derivative of the methyl

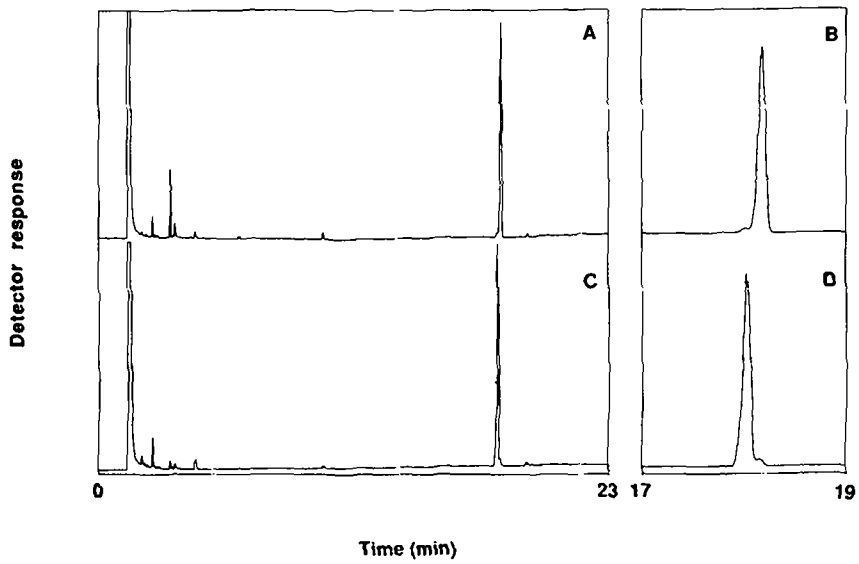

FIG. 3. Gas-liquid chromatographic analysis of the (−)-menthoxycarbonyl derivatives of dimethyl 2-hydroxydodecane-1,12-dioates obtained from methyl *threo*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoate (A,B) and from methyl *erythro*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoate (C,D). Conditions: methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm); carrier gas, helium; flow rate, 25 cm/s; injection port temperature, 260°C; column temperature, 230°C raised to 276°C at 2°C/min.

11-hydroxy-12-octadecenoate. The main peak was due to the MC derivative of dimethyl 2*S*-hydroxydodecane-1,12-dioate (**VIII**, Fig. 2; 3% or less of the derivative of the *R* isomer), demonstrating that the absolute configuration of C-11 of the parent epoxy alcohol was *S*. This configuration coupled with the relative stereochemistry of C-11/C-12 *(erythro)* and the configuration of the 12,13-epoxide group *(trans)* further showed that the absolute stereochemistry of the epoxy alcohol was methyl 11*S*-hydroxy-12*S*,13*S*-epoxy-9*Z*-octadecenoate. The *S* configuration established for C-13 was in agrement with the mode of formation of the epoxy alcohol from 13*S*-HPOD, *i.e.*, by a reaction in which the absolute configuration of C-13 was not affected (8).

Absolute stereochemistry of methyl threo-*11-hydroxy-trans-12,13-epoxy-9Z-octadecenoate.* Catalytic hydrogenation of methyl *threo*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoate (1 mg) yielded the corresponding saturated epoxy alcohol. An aliquot was analyzed as the Me_3Si derivative by GC/MS. The C value was 20.96 and the mass spectrum was similar to that of the corresponding derivative of hydrogenated methyl *erythro*-11-hydroxy-*trans*-12,13-epoxy-9*Z*-octadecenoate. Treatment of the hydrogenated *threo* epoxy alcohol with triphenylphosphine selenide and trifluoroacetic acid afforded methyl 11-hydroxy-12*E*-octadecenoate. The C value of the Me_3Si derivative of this compound was 19.77 and the mass spectrum was identical to that of the Me_3Si derivative of methyl 11-hydroxy-12-octadecenoate obtained from the *erythro* epoxy alcohol (see above). The infrared spectrum showed an absorption band at 970 cm^{-1}, confirming that the Δ12 double bond had the *E* configuration. Gas chromatographic analysis of the esterified product obtained following oxidative ozonolysis performed on the MC derivative of the allylic alcohol is shown in Figure 3 (A and B). A main peak due to the MC derivative of dimethyl 2*R*-hydroxydodecane-1,12-dioate appeared (3% or less of the derivative of the *S* isomer), demonstrating that the absolute configuration at C-11 of the epoxy alcohol degraded was *R*. This result, coupled with the relative stereochemistry of the epoxy alcohol, showed that its absolute stereochemistry was 11*R*-hydroxy-12*S*,13*S*-epoxy-9*Z*-octadecenoate.

Absolute stereochemistry of erythro-*hepoxilin* B_3. A sample of the methyl ester of *erythro*-hepoxilin B_3 (0.3 mg) was hydrogenated to afford methyl *erythro*-10-hydroxy-*trans*-11,12-epoxyeicosanoate. The mass spectrum of the Me_3Si derivative showed prominent ions at *m/e* 413 (M − 15, 15%), 381 [M − 47 (loss of $\cdot CH_3$ plus CH_3OH), 1], 287(13), 273 (Me_3SiO^+=CH-$(CH_2)_8$-$COOCH_3$, 100), 257 [M − 171 (loss of $\cdot(CH_2)_8$-$COOCH_3$), 26], and 169 (OHC-$(CH_2)_8$-C≡O^+, 25). Deoxygenation by treatment with triphenylphosphine selenide and trifluoroacetic acid afforded methyl 10-hydroxy-11-eicosenoate as judged by analysis of the Me_3Si derivative by GC/MS. Prominent ions in the mass spectrum were present at *m/e* 397 (M − 15, 2), 365 [M − (15 + 32), 3], 322 [M − 90 (loss of Me_3SiOH) 1], 299 [M − 113 (loss of $\cdot C_8H_{17}$), 6], and 241 (Me_3SiO^+=CH-CH=CH-C_8H_{17}, 100). The MC derivative of this hydroxyester was subjected to oxidative ozonolysis. The esterified product contained a major MC derivative, *i.e.*, that of dimethyl 2*S*-hydroxyundecane-1,11-dioate (Fig. 4, A and B; 3% or less of the derivative of the *R* isomer), demonstrating that C-10 of the parent hepoxilin B_3 had the *S* configuration. Because of the *erythro* relationship between C-10/C-11 and the fact that the 11,12-epoxide group had the *trans* configuration, it followed that the absolute stereochemistry of the hepoxilin B_3 degraded was 10*S*-hydroxy-11*S*,12*S*-epoxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid. This hepoxilin was isolated together with *inter alia* 12*S*-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (16). It seems probable that the compound was formed by a rearrangement analogous to that occurring in the formation of the above-mentioned *erythro*- and

METHOD

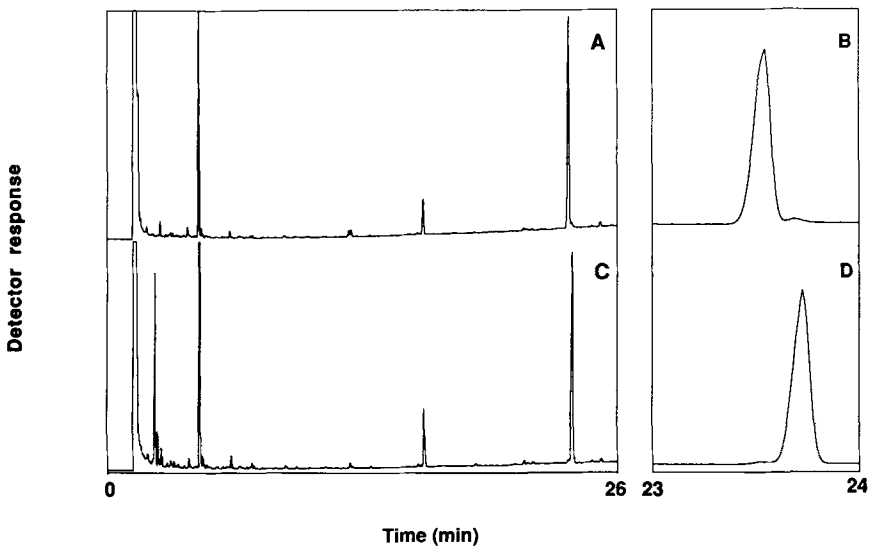

FIG. 4. Gas-liquid chromatographic analysis of the (−)-menthoxycarbonyl derivatives of dimethyl 2-hydroxyundecane-1,11-dioates obtained from the methyl ester of *erythro*-hepoxilin B_3 (A,B) and from the methyl ester of *threo*-hepoxilin B_4 (C,D). Conditions were the same as those given in Figure 3, except for the column temperature, which was programmed from 210°C to 262°C at 2°C/min.

threo-hydroxy-epoxy-octadecenoates from 13*S*-HPOD, *i.e.*, from 12*S*-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid by a process that did not affect the absolute configuration of C-12.

Absolute stereochemistry of threo-*hepoxilin* B_4. Catalytic hydrogenation of the methyl ester of *threo*-hepoxilin B_4 (0.2 mg) yielded methyl *threo*-10-hydroxy-*trans*-11,12-epoxyeicosanoate. Deoxygenation afforded methyl 10-hydroxy-11-eicosenoate, the MC derivative of which was subjected to oxidative ozonolysis. GLC analysis of the esterified product showed the presence of a major MC derivative, *i.e.*, the MC derivative of dimethyl 2*R*-hydroxyundecane-1,11-dioate (Fig. 4, C and D; 2% or less of the derivative of the *S* isomer). Thus, the parent hepoxilin B_4 had the *R* configuration at C-10 and, as deduced from the stereochemistry at C-10/C-11 and of the epoxide group, the absolute stereochemistry 10*R*-hydroxy-11*S*,12*S*-epoxy-5*Z*,8*Z*,14*Z*,17*Z*-eicosatetraenoic acid.

Conclusion. The present method for determination of the absolute stereochemistry of epoxy alcohols of type **I** (Fig. 1) is useful for steric analysis of, *inter alia*, hepoxilins of the B series from various sources. Hepoxilins B_3 and B_4 are formed from arachidonic and 5,8,11,14,17-eicosapentaenoic acids, respectively, *via* 12-hydroperoxide intermediates (7,21,22). Hepoxilin B_3 exerts biological effects in several systems, notably an insulin release-promoting effect in the rat (23). In addition, the method will be suitable for determination of the absolute stereochemistry of new *cis*-epoxy alcohols recently isolated following incubation of fatty acid hydroperoxides with flaxseed allene oxide synthase (24).

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Headspace Gas Chromatography to Determine Human Low Density Lipoprotein Oxidation

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We previously described a rapid headspace gas chromatographic method for the determination of hexanal, an important decomposition product of n-6 polyunsaturated fatty acid (PUFA) peroxidation in rat liver samples and human red blood cell membranes. This method was applied to the measurement of Cu^{2+} catalyzed-oxidation of freshly prepared human low density lipoproteins (LDL) from 10 healthy adult volunteers. A twofold variation in oxidative susceptibility was found by this assay for hexanal and other volatiles. Hexanal values correlated significantly ($P < 0.05$) with total polyunsaturated fatty acid (PUFA), 18:2 and n-6 PUFA contents of LDL; but poorly with 20:4 and with vitamin E. Therefore, in addition to α-tocopherol, other endogenous antioxidants and factors may contribute to LDL's resistance to oxidation. This simple, rapid and sensitive method for oxidative susceptibility provides a useful component in the analysis of the prooxidant/antioxidant status of biological samples. The method is used routinely in our laboratories to determine specific peroxidation products of n-6 and n-3 PUFA.
Lipids 27, **1047–1051 (1992).**

Modification of low density lipoproteins (LDL) by oxidation of its polyunsaturated fatty acid (PUFA) components appears to play a major role in atherosclerosis (1). Oxidative modification of LDL is promoted by arterial endothelial cells, smooth muscle cells and macrophages. Aldehyde decomposition products are formed by *in vitro* oxidation of LDL catalyzed by iron or copper ions (2–5). However, the *in vivo* origin of these metal catalysts has not been identified. The aldehydes produced by metal-catalyzed PUFA peroxidation are cytotoxic and may react with and derivatize the amino acid residues of the apoprotein B to generate modified LDL.

Cholesterol oxides and oxysterols formed in oxidized LDL appear to injure endothelial cells, promote build-up of plaques, are atherogenic and mutagenic, and inhibit cholesterol metabolism (6). Hydroperoxy and hydroxy fatty acid derivatives accounted for about 70% of the products formed from linoleate during *in vitro* oxidation of LDL in the presence of $CuSO_4$ (7). However, considerable variation was observed in the oxidative susceptibility of different LDL samples. LDL particles were suggested to contain some unidentified factor conferring susceptibility or resistance to oxidation (8).

The results of *in vitro* and *in vivo* studies to assess the effects of oxidation and antioxidation processes in LDL are difficult to interpret because inherently imprecise methodology has been used to measure lipid oxidation and the oxidative susceptibility of LDL to oxidation.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reacting substances; ultraviolet, UV.

Measurements of the susceptibility of LDL toward oxidative stress have been based on determinations of thiobarbituric acid-reacting substances (TBARS) (3,8–16), conjugated dienes (3,12–14,17,18), fluorescent products (3,13) and peroxide value (13,19–21). These methods are, however, complex assays that lack specificity and measure a great variety of compounds. Chemically more specific measurements also have been used to measure oxidative modification of LDL, including specific aldehydes by high-performance liquid chromatography (HPLC) (3,13) and products of cholesterol oxidation by gas chromatography (GC) (8), but these methods are too tedious for routine analyses of oxidative susceptibility. The loss of 18:2 and 20:4 has also been used to measure LDL oxidation (3,7), but this method is not sufficiently sensitive to follow early stages of oxidation. Other changes measured in modified LDL include electrophoretic mobility (8,13,15), degradation by macrophage and endothelial cells (9–11,13,15,20, 22), and cytotoxicity (16).

We recently described a rapid headspace gas chromatographic method for the determination of hexanal, an important volatile PUFA decomposition product, as an indicator of n-6 PUFA peroxidation in rat liver samples (23). By this method, no work-up is necessary, and clean samples of hexanal can be taken from the headspace above a biological specimen. This method was used to study the effects of dietary lipids and vitamin E on *in vitro* and *in vivo* lipid peroxidation in rat liver and kidney (24) and in tissues from rats induced by iron (25). This method was further improved for the determination of specific volatile peroxidation products of n-6 PUFA (pentane and hexanal) and n-3 PUFA (propanol), and applied to a study of the susceptibility of human red blood cell membranes to lipid peroxidation (26).

Although much work has been published on the biological properties of oxidized human LDL, the data on the oxidative susceptibility of different LDL samples are sparse. This paper describes the application of headspace GC to measure Cu^{2+}-catalyzed oxidation of freshly prepared human LDL. This simple, rapid and sensitive method may provide a useful complement to the measure of the prooxidant/antioxidant status of biological samples.

MATERIALS AND METHODS

Materials. Hexanal and propanol used as standards for the headspace GC analyses were purified by chromatography through a Waters Sep-Pak silica cartridge (Waters Associates, Milford, MA) (23). Copper sulfate (10 mM $CuSO_4$, Fisher Scientific, Fairlawn, NJ) solutions used as oxidant were prepared fresh daily.

Human LDL. Blood was collected after overnight fasting by venipuncture in ethylenediaminetetraacetic acid (EDTA) from normolipidemic healthy adult volunteers and centrifuged at 1500 × *g* at 4°C to prepare plasma. Plasma LDL was prepared by sequential density ultracentrifugation (27) in the presence of 0.01% EDTA. Prior to the

oxidation experiments, LDL was exhaustively dialyzed with deoxygenated phosphate-buffered (10 mM, pH 7.4) saline (100 mM) for 24 h. The final concentration of each LDL sample was diluted with phosphate-buffered saline (10 mM) to the same protein concentration (1 mg/mL LDL). One portion was stored at −5°C for later lipid extraction and GC analyses of fatty acid composition (28,29), and vitamin E analyses (30). Oxidative susceptibility was determined immediately by headspace GC.

Oxidative susceptibility by headspace capillary GC. The oxidative susceptibility of LDL was measured by headspace capillary GC (23,26) to detect low levels of aldehyde products and to distinguish between oxidation of n-6 (hexanal and pentane) and n-3 (propanal) PUFA at various oxidation levels. Volatiles were determined by capillary gas chromatograph with a headspace sampler (Perkin-Elmer Sigma 3B gas chromatograph with an H-6 headspace sampler, Norwalk, CT), a capillary DB-1701 column (30 m × 0.32 mm, 1 μm thickness, J&W, Folsom, CA) heated isothermally at 80°C. The GC conditions were the same as those used previously to measure hexanal in red blood cell membranes (26): capillary column, DB-1701; oven temperature, 80°C; injector, 180°C; detector, 200°C. Volatile compounds derived from the oxidation of LDL were identified by comparison of retention times with those of authentic reference compounds. Analyses were standardized daily with solutions of 10 μM hexanal.

Duplicate samples of 0.25 mL LDL in phosphate-buffered saline and standard solutions of hexanal were measured into special headspace 6-mL bottles sealed with silicone rubber teflon caps with a crimper and incubated in a water bath shaker for 1, 2, 4 and 24 h at 37°C in the presence of various levels of Cu^{2+} (2, 4, and 8 μM $CuSO_4$). After incubation, the bottles were inserted into the headspace sampler heated at 40°C, pressurized with carrier gas for 30 s, and an aliquot of gas phase (headspace) was injected directly into the gas chromatograph through the stationary injection needle.

Lipid analyses. Total lipids were extracted by the method of Burton *et al.* (29) using mixtures of ethanol and hexane in the presence of sodium dodecylsulfate (29). An aliquot of the lipid extract was methylated with acetyl chloride in methanol/benzene (4:1, vol/vol) (31) in the presence of triheptadecanoin added as internal standard. Fatty acid analyses of extracted lipids (28) were carried out by GC (Hewlett Packard, Avondale, PA, model 5890, equipped with a flame ionization detector) with a polar DB-23 capillary column (50% cyanopropylphenyl, J&W, 30 m × 0.25 mm) and identified by comparison with authentic standards (NuChek Prep, Elysian, MN).

Other determinations. The protein content of LDL samples was estimated by the method of Bradford (32). Vitamin E was determined by HPLC (30).

RESULTS

Volatile lipid oxidation products were measured by the headspace GC method directly on LDL samples without extraction. The addition of Cu^{2+} at different concentrations was very effective in catalyzing lipid peroxidation in human LDL as measured by the headspace GC method. A typical gas chromatogram of human LDL oxidized with 4 μM $CuSO_4$ is shown in Figure 1. Four main components were identified by comparison of retention times

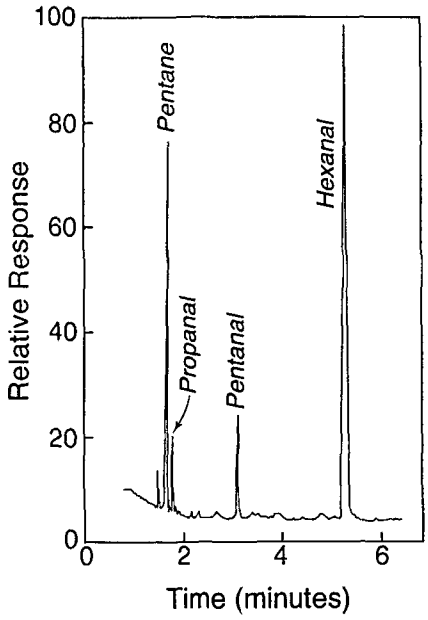

FIG. 1. Capillary gas chromatography of a 0.25-mL sample of human low density lipoprotein (LDL) (containing 1.0 mg protein/mL LDL) in 10 mM phosphate-buffered saline after incubation for 4 h at 37°C in the presence of 4 μM $CuSO_4$.

with those of authentic reference compounds, namely pentane, propanal, pentanal and hexanal. Pentane and hexanal are derived from oxidation of n-6 PUFA, and propanal from oxidation of n-3 PUFA (33). Pentanal is presumed to come from hexanal by formaldehyde formation in the presence of Cu^{2+} (33).

Table 1 shows the distribution of 4 volatiles in 10 different LDL samples oxidized with 2, 4 and 8 μM Cu^{2+} for different time periods at 37°C. Hexanal is the main volatile, followed by pentanal, pentane and propanal. Significant levels of volatiles were determined after incubation with 4 and 8 μM Cu^{2+} for 2 or 4 h. A wide range of volatiles were formed from the different LDL samples assayed. With 2 μM Cu^{2+}, 2.6 times more hexanal was produced after 4 h by LDL-F than LDL-D. With 4 and 8 μM Cu^{2+}, hexanal formation varied from 113 (LDL-B) to 371 nmol/mg (LDL-A) after 4 h. These results indicate that LDL from different individuals have a wide variation in oxidative susceptibility as measured by the headspace GC method.

Figure 2 shows time plots of hexanal formation obtained with 4 samples of LDL oxidized in the presence of 8 μM $CuSO_4$. These plots show a lag phase of about 1 h, followed by rapid production of hexanal, then slowing down between 4 and 24 h. The Cu^{2+}-catalyzed oxidation of LDL can thus be conveniently monitored by headspace GC of hexanal, which is the main decomposition product of hydroperoxides of n-6 PUFA.

Hexanal formation follows kinetics similar to those reported by Esterbauer *et al.* (17) for conjugated diene formation after a variable lag phase in LDL oxidized with 1.66 μM Cu^{2+}. Esterbauer *et al.* (17) used the duration of the lag phase as a measure of oxidative susceptibility of different LDL samples, and considered the lag phase to reflect the antioxidant status of the samples. By using the headspace GC method, more variation in hexanal

METHOD

TABLE 1

Formation of Volatile Products by $CuSO_4$ Oxidation of Human Low Density Lipoprotein (LDL) at 37°C[a]

Subjects	Treatment (μM Cu^{2+})	Incubation time (h)	nmol/mg LDL protein)			
			Pentane	Propanal	Pentanal	Hexanal
A	Control	4	0.0	0.0	0.0	0.0
	8	1	0.0	0.8	0.0	2.9
	8	2	8.7	2.3	15.9	163
	8	4	31.8	3.3	52.2	371
	8	24	54.2	6.7	144	452
B	Control	24	0.0	0.0	0.0	0.0
	4	2	5.4	0.9	0.0	4.7
	4	4	34.1	2.5	9.9	113
	4	24	51.2	4.5	95.7	466
	8	1	2.9	0.3	0.0	7.2
	8	2	66.8	3.0	17.5	156
	8	4	262	5.3	53.1	325
	8	24	105	8.5	225	530
C	2	2	2.5	1.6	1.7	5.1
	2	4	9.2	1.6	3.7	60.2
	2	24	32.2	3.1	48.7	323
	4	2	8.0	2.1	6.8	74.8
	4	4	33.1	3.4	31.5	262
	4	24	53.5	5.4	123	406
	8	2	23.4	3.0	14.0	149
	8	4	90.4	4.1	42.8	320
	8	24	50.0	5.4	118.6	340
D	4	4	34.2	5.2	52.4	390
	8	4	47.1	5.9	70.9	475
E	4	2	7.6	4.5	11.5	113
	4	4	37.1	7.7	52.5	343
	8	2	15.6	5.5	20.5	104
	8	4	59.2	9.3	86.1	438
F	2	4	9.6	4.2	14.4	154
	4	4	33.4	4.8	43.5	243
	8	4	34.9	5.6	48.8	287
G	4	2	4.1	2.2	8.3	98.9
	4	4	29.4	4.1	41.9	347
	8	2	4.7	2.8	10.0	129
	8	4	31.1	4.1	42.9	452
H	4	2	0.0	0.4	0.0	9.3
	4	4	0.0	1.2	0.0	5.2
	8	2	17.9	5.5	25.3	230
	8	4	40.2	5.1	49.9	347
I	8	2	1.6	2.4	4.8	74.2
	8	4	14.4	3.6	26.6	229
J	8	2	7.7	2.1	9.0	128
	8	4	30.5	3.5	40.5	350

[a]Determined by headspace capillary gas chromatography directly on 0.25 mL LDL (1 mg protein/mL LDL; see Materials and Methods). Average SD of duplicate analyses is 5.2%.

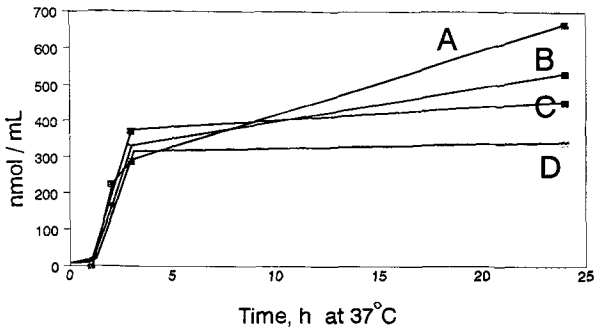

FIG. 2. Kinetics of hexanal formation in four different samples (A, B, C, D) of human low density lipoprotein oxidized for 24 h in the presence of 8 μM $CuSO_4$ (see Table 1 for data on other volatile oxidation products).

formation was observed after 2 and 4 h incubation with Cu^{2+}. Therefore, hexanal values reflect variation in n-6 PUFA content of LDL, as well as variation in the rate by which they decompose.

Variation in oxidative susceptibility was studied by determining the effect of Cu^{2+}-catalyzed oxidation on samples of freshly prepared human LDL from 10 healthy adult volunteers. The headspace GC assay for hexanal as a direct measure of LDL oxidative susceptibility was compared with the fatty acid composition and vitamin E contents of the corresponding lipid extracts (Table 2). Hexanal values correlated significantly ($P < 0.05$) with total PUFA, 18:2 and n-6 PUFA contents of LDL of eight subjects. There was no correlation, however, between hexanal formation and tocopherol contents and 20:4 in these LDL samples. These results indicate that dietary PUFA may be

TABLE 2

Formation of Hexanal as a Measure of Susceptibility of Human LDL to Lipid Peroxidation with Cu^{2+}[a]

Subjects	Hexanal (nmol/mg protein)	PUFA (Rel%)	18:2 (Rel%)	n-6 PUFA (Rel%)	20:4 (Rel%)	18:1 (Rel%)	Vitamin E (μg/dL)
A	371	43.5	32.3	42.4	8.6	19.7	780
B	325	40.6	28.3	39.1	8.8	18.5	709
C	320	43.6	29.2	40.5	9.4	13.4	665
D	475	44.5	33.1	42.1	7.6	17.4	643
E	438	40.9	29.2	39.7	8.5	19.6	1613
F	287	43.2	32.8	42.1	7.7	19.6	1053
G	452	47.2	37.8	45.2	5.4	17.5	1055
H	347	47.1	34.6	45.0	7.8	15.8	867
I	229	33.0	22.6	31.1	6.4	19.1	n.d.[b]
J	350	43.2	34.1	41.6	6.2	15.7	n.d.

Regression analysis:

	N	R	$P <$
Hexanal *vs.* PUFA	10	0.609	0.05
Hexanal *vs.* 18:2	10	0.596	0.05
Hexanal *vs.* n-6 PUFA	10	0.610	0.05
Hexanal *vs.* 20:4	10	0.479	n.s.[b]
Hexanal *vs.* vitamin E	8	0.448	n.s.

[a] Hexanal determined by headspace capillary gas chromatography after incubation of LDL for 4 h at 37°C in the presence of 8 μM $CuSO_4$. Abbreviations: LDL, low density lipoproteins; PUFA, polyunsaturated fatty acids; Rel, relative.

[b] n.d., Not determined; n.s., not significant.

one of the most important factors affecting the *in vitro* oxidative susceptibility of human LDL.

DISCUSSION

Oxidation of LDL and other lipoproteins in circulating blood has been implicated in the etiology of coronary heart diseases (1,13). Esterbauer *et al.* (3,13) discussed the impact that aldehydes produced by hydroperoxide decomposition have in the modification of LDL recognized by the scavenger receptor. Although the free aldehydes measured as dinitrophenylhydrazones after separation by thin-layer chromatography (TLC) and HPLC were minor products, they concluded that most of the aldehydes reacted with apo B and lipids to produce fluorescent materials.

Recent reports showed that the fatty acid composition of LDL is influenced by diets rich in oleic acid (12,14) and by supplementation of fish oil n-3 PUFA (34,35). Oleate-rich LDL particles from rabbits (12) and from humans (14) fed diets rich in oleic acid were highly resistant to oxidative modification based on formation of TBARS, conjugated dienes and cell induced oxidation by incubation with endothelial cells. However, since oxidation of oleic acid does not produce TBARS (36) and conjugated dienes (37), these assays would not be appropriate to test oxidative susceptibility of oleate-rich LDL.

Oxidative modification of LDL from smokers fed fish oil resulted in significantly higher TBARS, compared to baseline values, than in nonsmokers (34). In another study (35) the susceptibility to Cu^{2+}-catalyzed peroxidation as measured by TBARS was similar in LDL from human groups on fish oil and corn oil diets. Therefore, our results agree with those of Jessup *et al.* (20), who reported no correlation between the rate of peroxidation and tocopherol contents in LDL of five subjects. In another study, Esterbauer *et al.* (13,18) found no correlation between the oxidation resistance (as measured by the lag phase in Cu^{2+}-induced LDL oxidation) and the α-tocopherol content of human LDL. Clearly vitamin E content of LDL is not the sole determinant and not necessarily predictive of the oxidative susceptibility of LDL. Therefore, in addition to the *in vivo* antioxidant activity of α-tocopherol, other endogenous antioxidants or inhibitors may contribute to the resistance of LDL to oxidation. Our results are also consistent with previous observations that LDL from individuals have varying and characteristic susceptibilities to oxidative modification, as measured by electrophoretic mobility, TBARS and oxysterol formation (8), and by the lag phase of conjugated diene formation (13,18).

Studies of dietary, genetic and pathological modification of LDL need to be undertaken and will require sensitive, chemically distinctive and practical methods. However, much of the methodology used to investigate LDL oxidation yields results that are difficult to interpret. The conjugated diene method of Esterbauer *et al.* (17), and several variations of the thiobarbituric acid (TBA) test have been used most commonly to determine oxidation in LDL. The validity of these methods have been questioned by Halliwell and Gutteridge (38). Although diene conjugation is a useful method to measure oxidation in pure lipids, it cannot be used directly on biological samples, especially human body fluids that contain substantial amounts of ultraviolet (UV)-absorbing material. The rapid UV conjugated diene method developed by Esterbauer *et al.* (17) has been used extensively to make continuous measurements of LDL oxidation and to determine duration of the lag phase as a sensitive

method for oxidative susceptibility. However, a recent study from the same group (21) compared conjugated diene with peroxide content by an iodometric assay in oxidized pig LDL. They found ratios of peroxides to conjugated dienes varying from 2.33 to 2.79 in oxidized LDL subfractions, and concluded that non-UV absorbing peroxides are formed besides conjugated hydroperoxides.

The validity of the TBA determination as an index of lipid peroxidation in biological samples has been a matter of considerable debate for some time. The determination of TBARS inherently lacks specificity and is flawed by analytical artifacts because it is affected by the same factors as lipid peroxidation (39). Factors reported to affect the color formation in the TBA reaction include heating temperature and time, pH, and the presence of metal-ion catalysts and of antioxidants (40). Because the TBA method is open to interference by many substances, including compounds that do not derive from lipid peroxidation, careful corrections are necessary through use of appropriate blanks (41). With its lack of specificity, the TBA test is not recommended for human biological materials (38,42).

The headspace GC method, used in this study to determine hexanal, provides a useful tool to measure the effect of Cu^{2+}-catalyzed oxidation on samples of freshly prepared human LDL. Because this method measures specific volatile aldehydes, it not only provides information on the degree of hydroperoxide decomposition but can distinguish between products of n-6 PUFA (pentane, hexanal) and n-3 PUFA (propanal) oxidation. The method can thus complement the conjugated diene absorbance method, which measures the effect of antioxidants on initial hydroperoxide formation. The method is sensitive and is used routinely in our laboratories to determine specific peroxidation products of n-6 and n-3 PUFA, and the prooxidant/antioxidant status of biological samples. Because the headspace technique is simple and rapid, we are now able to analyze biological samples with a throughput of 48 determinations per day. This feature of our assay is essential in analyzing replicate fresh samples and in minimizing storage that would affect results of oxidative susceptibility. More research is needed to elucidate factors that influence the balance between antioxidant and prooxidant factors that affect the susceptibility of LDL to oxidation.

ACKNOWLEDGMENTS

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Fluorescence Assay of Glucosylceramide Glucosidase Using NBD-Cerebroside

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A sensitive fluorometric assay for glucocerebroside β-glucosidase [Dinur, T., Grabowski, G.A., Desnick, R.J., and Gatt, S. (1984) *Anal. Biochem. 136*, 223–234] has been reexamined. It was found that the lipids containing the NBD moiety (12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)] used for standardization of the assay are light-sensitive and that the yield of fluorescent light is very sensitive to the composition of the solvent used in the fluorometric measurement. Some protection against fading could be obtained by adding a free-radical trapping agent, SlowFade. The fading of the free NBD-acid, when used for standardization, could be prevented by adding ethanol to the solvent, but this reduced the fluorescence yield. It is recommended that some of the fluorescent substrate be enzymatically hydrolyzed completely to NBD-ceramide, which can be utilized as the standard without the need to add ethanol. A warning about enzyme reaction rate stability with time is given, with a suggestion for ensuring constancy of activity.
Lipids 27, 1052–1054 (1992).

A simple, sensitive and useful method for the assay of glucosylceramide β-glucosidase (EC 2.3.2.45) has been described (1,2), in which a fluorescent analog of glucosylceramide (NBD-GlcCer) is used as the substrate and the fluorescent enzyme product (NBD-ceramide) is separated from the substrate by liquid/liquid partitioning. While other useful methods are also available, this one has the advantages of simplicity, commercial availability of the substrate and elimination of the need for radioactivity. However, there is a problem in converting the fluorescence readings to molar amounts, since the substrate is not soluble in the solvent used for reading. The authors (2) suggest that a standard solution of NBD-ceramide or NBD-dodecanoic acid be used instead.

Two solvent partitioning steps are used to isolate the product of enzyme action. The first involves the incubation mixture (0.45 mL of water), 0.45 mL of isopropyl alcohol, and 1.5 mL of heptane. NBD-ceramide enters the upper phase, together with a little of the substrate; the latter is removed by transferring most of the upper phase, possibly 1.4 mL, and partitioning it against 0.35 mL of water. The first upper phase (called "theoretical upper phase", TUP) differs in composition from the second, final upper phase (FUP).

The earlier authors (2) calculated the amount of enzymatically-formed ceramide by measuring the fluorescence of a known amount of NBD-ceramide or NBD-acid in the first upper phase, although FUP is the solvent actually used in the fluorometric measurement. The statement is made that the two compounds yield the same molar fluorescence values.

No comment was offered about the possible bleaching by light of the NBD compounds during handling and reading, although fluorescent compounds are notorious for fading under some conditions.

In our utilization of the NBD method, we found some discrepancies that led us to investigate the method in more detail. This note describes changes in the procedure that improve the reliability of the method.

MATERIALS AND METHODS

NBD-GlcCer, NBD-dodecanoic acid, mixed ceramides (Type III) from brain sphingomyelin, and Na taurocholate were from Sigma Chemical Co. (St. Louis, MO). SlowFade was from Molecular Probes, Eugene, OR (gift of Elizabeth Marcus). GlcCer was prepared from a Gaucher spleen (3).

Homogenized Madin-Darby canine kidney cells were used as the glucosidase source. The substrate emulsion in Triton X-100 + Na taurocholate and the incubation mixture were prepared as described (2), and the resultant fluorescent ceramide was assayed as described in a 1-mL cuvet in a Perkin-Elmer (Norwalk, CT) Luminescence Spectrometer LS-5B, with excitation at 460 nm and emission at 515 nm. A relatively large batch of NBD-ceramide was prepared by scaling up the standard incubation and partitioning procedures, using a 20-h incubation at 37°C to hydrolyze all of the substrate.

By similar scaling up, TUP was prepared with water, isopropyl alcohol and heptane (15:15:50, vol/vol/vol) in a separatory funnel. FUP was prepared from the upper layer.

RESULTS

Portions of 2 μM NBD-acid in FUP were stored under fluorescent laboratory light for 10, 40 and 70 min, yielding readings of 90, 70 and 57 units, respectively. Similar instability was found also with more dilute solutions. Thus it was evident that the acid was unstable in light after final partitioning. When NBD-acid was stored under the same conditions in absolute ethanol-FUP solutions, all the solutions were stable to light even with as little as 10% ethanol. The wavelengths of maximal excitation and emission in 100% ethanol were shifted to 475 and 535 nm, and there was a small loss of fluorescence yield (74 units for 2 μM acid).

Further study with 190 nM NBD-acid in test tubes stored 15 to 120 min in FUP or FUP/EtOH (90:10 vol/vol) showed that the readings with FUP could be brought up to the FUP/EtOH readings by adding the EtOH just before the readings were made. For example, NBD-acid stored in the dark for 15 min in FUP yielded 19 units, stored in FUP/EtOH they yielded 56 units and the addition of EtOH to FUP solutions yielded 55 units. Under these conditions, it appears that the NBD-acid solution, if used as a standard, should contain ethanol.

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Abbreviations: FUP, final upper phase; GlcCer, glucosylceramide; NBD-acid, 12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminododecanoic acid; NBD-GlcCer, the cerebroside made by acylating glucosylsphingosine with NBD-acid; TUP, theoretical upper phase.

METHOD

The fluorometric readings, with 462/523 nm settings in the fluorometer and NBD-acid in FUP/EtOH (90:10), were proportional to concentration between 25 and 400 nM, with the extrapolated line passing through the origin. At the 400 nM level, omitting the ethanol yielded a reading 78% lower.

Study of 100 nM NBD-ceramide in FUP or FUP/EtOH showed a somewhat different relationship. The ceramide in FUP, read at 515 nm, yielded 96.5, 101.5 and 103.5 fluorescence units after 15, 60 and 120 min of exposure to ambient fluorescent light, respectively. However, in FUP/EtOH (90:10, vol/vol), read at 523 nm, ceramide yielded much lower values: 38.1, 39.8 and 40.0 units for the same time periods. Thus, while ethanol stabilized the readings over time here too, it quenched the fluorescence from ceramide. Both solvents yielded readings that were proportional to ceramide concentration between 50 and 250 nM.

SlowFade, a proprietary free-radical scavenger solution, was tested to see if it exerted a stabilizing action. The reagent was included in the aqueous phase, using 5.6% of the total water, and the first upper phase was then prepared by adding heptane and isopropyl alcohol. FUP was prepared from this TUP and a 1-mL portion of each solution was added to 190 pmol of NBD-ceramide or acid. The readings, made after 30 min storage in ambient light (Table 1), showed, first, that there was a great difference in fluorescence when the two upper phases were compared, especially with NBD-ceramide. Second, the two fluorescent compounds responded differently to the two solvents. Third, SlowFade was only slightly useful in the case of ceramide (11% more fluorescence). Inclusion of SlowFade in the incubation system interfered with glucosidase activity.

The difference between the two fluorescent compounds with regard to the effect of the second partitioning step (decreased fluorescence with the acid *vs.* increased fluorescence with ceramide) is probably due to dimerization of the acid in the less polar FUP, and consequent self-quenching.

A point which does not seem to have been considered before is the possibility that the fluorescent yields might be affected by the detergents in the enzyme incubation solutions. We prepared TUP and FUP using an equeous system similar to the enzyme medium (Triton X-100 and Na taurocholate in phosphate-citrate buffer), as well as plain water. Results similar to those in Table 1 were obtained, and the detergents were found to make no significant difference in the readings with NBD-ceramide or NBD-acid. In this experiment, a slight amount of self-quenching was found with 250 nM ceramide and much more with the acid (*i.e.*, the reading with 250 nM NBD-acid in FUP was only 2.3 times the reading with 50 nM acid).

Another question needing study was the possibility that the presence of ceramide in the incubation system (due to enzyme action) affects the partition constant of the still-unhydrolyzed GlcCer. A test with the standard assay mixture (2), containing NBD-GlcCer and nonfluorescent GlcCer, was compared with the same mixture containing also 5 μg of ceramide. The readings obtained in the resultant FUP were very low and similar in the two samples. Evidently the two lipids do not form an extractable complex under these conditions.

TABLE 1

Effect of an Anti-Fade Reagent on the Fluorescence of NBD Lipids in the Upper Phases Obtained by the Two Partitioning Steps in NBD-Ceramide Isolation[a]

	Fluorescence intensity at 515 nm			
	NBD-ceramide		NBD-dodecanoic acid	
Solvent SlowFade present	No	Yes	No	Yes
First upper phase	88	100	75	75
Final upper phase	203	225	45	42

[a]The values shown are the means of duplicates.

DISCUSSION

In view of the difficulty of making an accurate comparison between free NBD-acid and NBD-ceramide molar light yields, and the incomplete constancy of the fluorescence/concentration ratio of the acid, it is preferable to use the ceramide as the standard. Because of the high cost of the ceramide, and since only a small amount of NBD-ceramide is needed to standardize each set of assays, it is much more economical to make it enzymatically from the substrate. β-Glucosidase, in the form of crude lysosomes, is readily prepared from any tissue by differential centrifugation. We incubated NBD-GlcCer at 37°C overnight in the standard incubation medium (2) and then isolated the product by partitioning in the usual way between heptane, isopropyl alcohol, and water. It is not necessary to purify the NBD-ceramide further since the background fluorescence from the reagents and enzyme is negligible. A thin-layer chromatogram of the material in the FUP, with a silica gel plate and chloroform/HOAc (90:10, vol/vol), examined under ultraviolet light, showed virtually complete conversion of the substrate, with just a trace of free NBD-acid. The concentrations of stock substrate and product solutions were determined by measuring at $\lambda = 475$ nm, rather than at 485.

In summary, our recommended changes in the procedure are (i) to make and use NBD-ceramide as the molar standard rather than NBD-acid, (ii) to standardize the assay with NBD-ceramide in FUP, not in TUP, and (iii) to handle the samples in reduced light as much as possible. Moreover, the sensitivity of the fluorescence yield to solvent composition emphasizes the need to do the partitioning and upper phase transfer with maximal reproducibility, preferably with a suction transfer device.

A weakness in the published method (2) is that the reaction rate, using human granulocyte extracts, was not constant over time. The initial rate was relatively high, then it slowed down and became constant after the first 30 min. This makes it difficult to calculate K_m and V_{max} values accurately. While it might be satisfactory to use short incubation times, when the linearity problem is not so severe, this weakens the sensitivity of the assay, since much higher readings can be obtained at the cost only of some patience. Another approach, also unsatisfactory, is to double the number of assay tubes and measure the amount of ceramide formed at the 30-min point and at a somewhat later point.

Perhaps an explanation for the changing velocities lies in a report that the human spleen contains two different GlcCer glucosidases (4). Later study (5) showed that one

form could be converted into the other, so it is posssible that the conversion occurs during incubation and that the final equilibrium mixture of enzymes is less active than its initial mixture. An early study in the laboratory of the senior author (NSR) yielded a very similar biphasic velocity activity curve with β-glucosidase from human placenta; preincubation with taurocholate eliminated the first velocity phase (6). Thus systems showing a biphasic time course should be tested with a preincubation protocol.

A study of the literature discloses several papers that give K_m and V_{max} values for this enzyme but very few of these make the claim that the reaction velocity was constant. Using Madin-Darby canine kidney cells, we found the rate was constant over time at all time points studied (5 to 60 min) with a K_m of 0.20 mM. Similar constancy was reported for porcine epidermis (7), mouse kidney (8), and purified enzyme from ox brain (9). Glucosidase from human tissues was said to yield constant activities in the case of placenta (10) and, for at least 60 min, with spleen membranes (11), and for 6 h with skin fibroblasts (12). At present, we can only urge investigators measuring β-glucosidase to check their velocity constancy.

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COMMUNICATION

Heterogeneity of Molecular Weight and Apolipoproteins in Low Density Lipoproteins of Healthy Human Males[1]

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The molecular weights of five low density lipoprotein (LDL) subfractions from four normal healthy males were determined by analytic ultracentrifuge sedimentation equilibria. Protein content of each subfraction was determined by elemental CHN analysis, and weights of apoprotein peptides were calculated. Molecular weights in subfractions of increasing density were 2.92 ± 0.26, 2.94 ± 0.12, 2.68 ± 0.09, 2.68 ± 0.28 and 2.23 ± 0.22 million Da, and protein weight percentages were 21.05, 21.04, 22.05, 23.10 and 29.10, in subfractions 1, 2, 3, 4 and 5, respectively. Total mean apoprotein weights for respective subfractions were 614 ± 53, 621 ± 45, 588 ± 9, 637 ± 83 and 645 ± 62 KDa. In addition to a single apoprotein B-100 (apo B-100) peptide with a mean carbohydrate content of 7.1% and a molecular weight of 550 KDa per LDL particle, there may be one or more apoprotein E peptides of 34 KDa and/or apoprotein C-III of 9 KDa. In addition, subfractions 4 and 5 may contain 3–7% apolipoprotein (a). There is considerable heterogeneity among LDL subfractions as well as within the same fraction from different individuals. This heterogeneity may relate to differences in origin, metabolism and/or atherogenicity as a result of their content of apoproteins other than apo B-100.
Lipids 27, 1055–1057 (1992).

Apolipoprotein B-100 (apo B-100) is the major apolipoprotein of very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). This apo B-100 is synthesized primarily in the liver and plays a pivotal role in lipoprotein metabolism as the LDL ligand that interacts with the LDL receptor and initiates receptor-mediated endocytosis and LDL catabolism. Chen *et al.* (1), Knott *et al.* (2), Yang *et al.* (3) and Law *et al.* (4) determined the complete 4536 amino acid sequence of the apo B-100 peptide, calculating a molecular weight of apo B-100 of 512,723 to 514,000 Da. La Belle and Krauss (5) found that 7.1% carbohydrate is associated with LDL apolipoproteins. While LDL contains predominantly apo-B, Campos and McConathy (6), Lee and Alaupovic (7) and Chapman *et al.* (8) observed the presence of apoprotein E and apoprotein C-III in the LDL density range.

The present study was undertaken to determine the accurate molecular weight of five LDL subfractions (1.0267–1.0492 g/mL) and the total mass of the apolipoproteins of these subfractions.

[1]Presented in part at the 78th AOCS Annual Meeting held in New Orleans, LA, May 1987; received best presentation award.

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Abbreviations: apo B-100, apo LP, apolipoprotein; apolipoprotein B-100; EDTA, ethylenediaminetetraacetic acid dipotassium salt; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

MATERIALS AND METHODS

Four healthy normolipoproteinemic male subjects were selected for this study. Subjects were fasted overnight. Blood was drawn into evacuated tubes containing ethylenediaminetetraacetic acid dipotassium salt (EDTA), 130 mg/100 mL, and centrifuged at 1300 × *g* for 20 min at 4°C to obtain plasma. The antimicrobial agent garamycin (7.5 mg/100 mL) and the protease inhibitor ε-amino caproic acid (130 mg/100 mL) were added. The plasma density was adjusted to 1.019 g/mL by mixing 4 mL plasma with 2 mL of a NaBr solution of density 1.0426 g/mL containing 0.196 moles of NaCl and 10 mg/100 mL EDTA. The resulting 6-mL solution(s) were centrifuged at 17°C in a 40.3 Beckman (Palo Alto, CA) rotor at 40,000 rpm for 18 h. The top 1 mL VLDL-IDL fraction was removed, and the second 1 mL was taken as background. The subnatant 4 mL was adjusted to 1.0670 g/mL by adding 2 mL of a NaBr solution, 1.1566 g/mL, containing 0.196 moles NaCl and 10 mg/100 mL EDTA. Again these tubes were similarly centrifuged for 24 h, and the top 1-mL fraction containing total LDL was recovered (9). Five LDL subfractions with densities 1.0267, 1.0306, 1.0358, 1.0422 and 1.0492 g/mL, respectively, were isolated by density gradient preparative ultracentrifugation (Model L8, Beckman) using a Beckman SW 45 Ti rotor (10). LDL subfractions were dialyzed overnight at 4°C against NaCl, 1.0063 g/mL, pH 7.2–7.4. Meniscus depletion equilibrium fringes were obtained after 72 h at 6704 to 9442 rpm in a Beckman Model E ultracentrifuge using a Rayleigh interference optical system for sedimentation equilibrium measurements (11). Air in the samples was displaced by N_2 in all the procedures in order to minimize oxidation. Apoprotein mass was determined in delipidated subfractions by CHN analysis. Procedures of the determination of partial specific volumes and CHN mass analysis have been described previously (12). The partial specific volume ($\bar{v}$), mL/g, of LDL subfractions was determined according to the equation given by Schachman (13):

$$\bar{v} = 1/\varrho_o - 1/x\{(\varrho-\varrho_o)/\varrho_o\} \quad [1]$$

where ϱ_o = solvent density (g/mL); ϱ = lipoprotein solution density (g/mL); x = mass concentration of LDL solution (g/mL).

Each lipoprotein subfraction together with an aliquot of its corresponding equilibrium gradient background salt solution was dialyzed (3×, 24 h each) in a 250-mL cylinder at 4°C against 1.0063 g/mL NaCl solution (10 mg/dL EDTA, pH 7.2–7.4). The CO_2 present in the dialysate was displaced by bubbling N_2 through it before use. Solvent and solution densities were measured to ± 0.000001 g/mL using an Anton-Paar DMA-60 sixth place density meter (Anton-Paar, Richmond, VA). The temperature of the density measuring cell was stabilized at 20 ± 0.001°C. The reference standards used for the density calibrations were dry air (corrected for barometric pressure) and purest

deionized water (boiled for 10 min to remove dissolved CO_2 and cooled to 20°C). The mass (g/mL) of each LDL solution was determined to ± 0.5% using a modified CHN analyzer (Model 185, Hewlett-Packard, Palo Alto, CA). Elemental CHN determinations were carried out in triplicate, sedimentation equilibrium runs in duplicate. Considering all experimental uncertainties a molecular weight of 2.68 million Da can be determined with an error of ± 40 KDa (± 1.5%). This amounts to an 8-12 KDa error in the molecular weight of the apolipoprotein depending upon the protein content of a particular subfraction. In reality, the errors may be considerably smaller.

RESULTS AND DISCUSSION

The molecular weights of five LDL subfractions from four healthy male subjects, as determined by sedimentation equilibrium analysis, ranged from 1.70 to 3.61 million Da (Table 1). The mean molecular weights for subfractions 1, 2, 3, 4 and 5 were 2.92 ± 0.26, 2.94 ± 0.12, 2.68 ± 0.09, 2.68 ± 0.28 and 2.23 ± 0.22 million Da, respectively. The mean protein content expressed as wt% of LDL was 21.05, 21.04, 22.05, 23.10 and 29.10 for subfractions 1, 2, 3, 4 and 5, respectively (Table 2). Calculated mean molecular weights of apoprotein for LDL subfractions 1, 2, 3, 4 and 5 were 614 ± 53, 621 ± 45, 588 ± 9, 637 ± 83 and 645 ± 62 KDa, respectively (Table 3).

The mean molecular weights of the five LDL subfractions analyzed in this study (Table 1) tended to decrease with increasing density and decreasing flotation rate of the subfractions. We previously reported that molecular weights of LDL subfractions did not consistently decrease with increasing density (14). The different result is probably due to the small number of subjects involved in these studies. In the current study the molecular weights of the LDL subfractions of individual subjects did not consistently decrease with increasing density. There was a different subfraction in each of the four subjects showing the highest molecular weight. In general, protein as weight percent of LDL increased as the density of LDL subfractions increased (Table 2). The total apolipoprotein contents of subfractions 1 and 2 were similar, but increased by 38% from fraction 2 to fraction 5.

The total apoprotein associated with various LDL subfractions had molecular weights ranging from 475 to 841 KDa (Table 3). Since a single molecule of apolipoprotein B-100 with a peptide weight of 514 KDa (1-4) is associated with each LDL particle (15), the two subfractions 6622 No. 1 and 7222 No. 2 with peptide weights of 502 and 475 KDa, respectively, appear to contain only apoprotein B-100. The low molecular weight suggests either that the apo-B in these fractions is not glycosylated or, alternatively, that these fractions may contain truncated apo B-100. Truncated forms of apo B such as apo B-37, apo B-40, apo B-54.8 and apo B-90 have been reported (16-18).

Since certain fractions contain more apoprotein than could be contributed by apo-B, it is possible that LDL can contain additional apoproteins. Since a single molecule of apo B-100 weighs 550 KDa including its carbohydrate component, a mean of 12, 13, 7, 16 and 17 wt% of subfractions 1, 2, 3, 4 and 5, respectively, may be comprised of other apoproteins. These other apoproteins could consist of apo E with a molecular weight of 34 KDa and/or apo C-III of 9 KDa (8). Since we did not determine the

TABLE 1

Molecular Weight[a] of Low Density Lipoprotein (LDL) Subfractions from Four Healthy Human Males Based on Sedimentation Equilibrium[b]

	LDL subfraction[c]				
Subject	1	2	3	4	5
6622	2.41	2.98	2.62	3.40	2.54
6769	2.95	3.28	2.45	2.81	2.65
7222	3.61	2.73	2.80	2.09	2.02
7384	2.70	2.78	2.83	2.41	1.70
Mean ± SEM	2.92 ± 0.26	2.94 ± 0.12	2.68 ± 0.09	2.68 ± 0.28	2.23 ± 0.22

[a]Million daltons.
[b]6704-9442 rpm, 20.0°C.
[c]Subfractions 1, 2, 3, 4 and 5 correspond to LDL of density of 1.0267, 1.0306, 1.0358, 1.0422 and 1.0492 g/mL, respectively.

TABLE 2

Weight % Protein[a] in Low Density Lipoprotein (LDL) Subfractions in Four Healthy Human Males

	LDL subfraction[b]				
Subject	1	2	3	4	5
6622	20.84	20.30	22.24	24.73	31.04
6769	21.75	22.83	23.55	24.86	26.65
7222	20.74	21.43	21.95	22.71	27.45
7384	20.86	19.58	20.47	20.09	31.24
Mean ± SEM	21.05 ± 0.24	21.04 ± 0.71	22.05 ± 0.63	23.10 ± 1.12	29.10 ± 1.19

[a]Protein by elemental CHN analysis.
[b]For density of the subfractions see Table 1.

TABLE 3

Total Molecular Weight[a] of Apolipoproteins in Low Density Lipoprotein (LDL) Subfractions in Four Healthy Human Males

	LDL subfraction[b]				
Subject	1	2	3	4	5
6622	502	605	583	841	788
6769	642	749	577	699	706
7222	749	585	615	475	554
7384	563	544	579	532	531
Mean ± SEM	614 ± 53	621 ± 45	588 ± 9	637 ± 83	645 ± 62

[a]Calculated from the data in Tables 1 and 2 and reported as KDa.
[b]For density of the subfractions see Table 1.

apo C-III or apo E content of these subfractions, we can only speculate on the relative amounts of apo E and/or apo C-III in these LDL subfractions. Lee and Alaupovic (7) reported apoprotein C-III and apo E ratios of 3.67, 1.91 and 3.09 for their subfractions designated 2, 3 and 4, respectively, which appear to correspond quite closely to fractions 1, 3 and 5 in this study. Only our fraction 1 with a possible apo C-III to apo E ratio of 3.0 may be considered close to the molar ratio of 3.09 reported for their subfraction 4 (7). Chapman *et al.* (8) reported one apo E per 60 LDL particles; however, from our data it is possible to have no apo E per LDL to three apo E per LDL molecule. Fractions 4 and 5 may also contain 3–7% as apolipoprotein (a) as previously suggested (8,19).

The current study demonstrates that there is considerable heterogeneity in molecular weight and apoprotein content among LDL subfractions as well as among the same subfraction from different individuals. The data strongly suggest that in addition to one apo B per LDL molecule there are variable amounts of smaller apoproteins, probably apo E and apo C-III. Subfractions of LDL may have different origins, metabolism and/or atherogenicity based on their content of apoproteins other than apo B-100.

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Effects of a Platelet-Activating Factor Antagonist, CV-6209, on Gastric Mucosal Lesions Induced by Ischemia-Reperfusion[1]

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Recent research was shown that oxygen-derived free radicals are involved in the pathogenesis of various diseases, including ischemia-reperfusion injury. We have also reported that oxygen-derived free radicals and lipid peroxidation may play an important role in gastric mucosal injury induced by ischemia-reperfusion. The hypoxanthine-xanthine oxidase system and neutrophils are considered important sources of oxygen-derived free radicals in this process. In recent years, it also has been shown that serum platelet-activating factor (PAF) levels increased during ischemia-reperfusion, and that induction of superoxide generation by neutrophils is one of the important biological effects of PAF. In the present study, we examined the effect of CV-6209, a specific PAF receptor antagonist, on gastric mucosal injury induced by ischemia-reperfusion, to shed some light on the possible involvement of PAF in such lesions. CV-6209 significantly attenuated the gastric mucosal injury induced by ischemia-reperfusion, and inhibited both an increase of thiobarbituric acid reactive substances and a decrease of α-tocopherol in gastric mucosa after ischemia-reperfusion. However, CV-6209 had no effect on gastric mucosal blood flow during ischemia-reperfusion. These results suggest that endogenous PAF may play an important role in gastric mucosal injury induced by ischemia-reperfusion, and that CV-6209 exerts its beneficial effect mainly by inhibiting neutrophil superoxide production induced by PAF.
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Platelet-activating factor (PAF) is an endogenous phospholipid that is produced by a variety of activated cells including neutrophils, basophils, macrophages, monocytes and endothelial cells (1–3). PAF exerts a wide spectrum of biological effects, such as stimulation of platelets and leukocytes, hypotension and bronchoconstriction, as well as an increase in vascular permeability (4). Considerable attention has recently been focused on PAF's potent ulcerogenic activity. PAF appears to be a mediator of endotoxin-induced gastrointestinal damage (5) and of gastric mucosal lesions induced by water-immersion and constriction (6). However, the precise mechanisms of PAF's ulcerogenic activity remain unknown. Oxygen-derived free radicals have been implicated in mediating tissue damage observed after ischemia in various organs (7). It also is known that PAF is synthesized by various cells under hypoxia (8) and that neutrophils and macrophages stimulated by PAF produce oxygen-derived free radicals (9). In the present study, we examined the effect of CV-6209, a PAF antagonist, on gastric mucosal injury induced by ischemia-reperfusion in rats. The experiments were intended to shed some light on the possible involvement of endogenous PAF in such lesions.

MATERIALS AND METHODS

Male Sprague-Dawley (SD) rats weighing 200 g were obtained from Keari Co., Ltd. (Osaka, Japan). Acute gastric mucosal injury was induced by using the technique previously described (10). After an 18-h fast, a laparotomy was performed on SD rats under pentobarbital anesthesia (25 mg/kg) and the celiac artery was blocked with a clamp for 30 min, followed by 30-min reperfusion upon release of the clamp. The extent of gastric mucosal lesions was carefully examined macroscopically and microscopically, and expressed in terms of the total area of the erosions (10). Thiobarbituric acid (TBA) reactive substances, as an index of lipid peroxidation, in serum and gastric mucosa were measured by the method previously reported (11,12), and serum α-tocopherol and gastric mucosal α-tocopherol were measured by the method of Abe *et al.* (13). α-Tocopherol is an excellent antioxidant which reacts with free radicals and terminates free radical-mediated chain reactions (14); hence, it is often used as an index of free radical involvement in the lesion. The PAF antagonist, CV-6209, which is 2-[*N*-acetyl-*N*-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl)aminomethyl]-1-ethylpyridinium chloride (Takeda Chemical Industry, Osaka, Japan) was dissolved in saline with 0.25% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) added, and was given to rats intravenously 10 min before clamping. Because CV-6209 is a surfactant, the solution was injected over a 5-min period to avoid hemolysis (15). The control group was treated with BSA-supplemented saline only. The microcirculatory blood flow in the gastric mucosa was measured using a laser Doppler flowmeter (16) (ALF 2100, Advance Co., Ltd., Tokyo, Japan).

Data are expressed as the mean ± SE of seven rats. Comparisons between groups were by the Dunnet test. With all statistical analysis, a probability (*P* value) of <5% was considered significant.

RESULTS

Clamping the celiac artery decreased the gastric mucosal blood flow to about 10% of that measured before the clamping. Just after the removal of the clamp, the blood flow in the gastric mucosa recovered completely. The decrease of blood flow during ischemia was not affected by treatment with CV-6209 at a dose of 10 mg/kg (Fig. 1).

Multiple erosions and bleeding were revealed in the gastric mucosa after 30 min of reperfusion following 30 min of ischemia. The total area of erosions induced by

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Abbreviations: BSA, bovine serum albumin; PAF, platelet-activating factor; SOD, superoxide dismutase; SD, Sprague-Dawley; TBA, thiobarbituric acid.

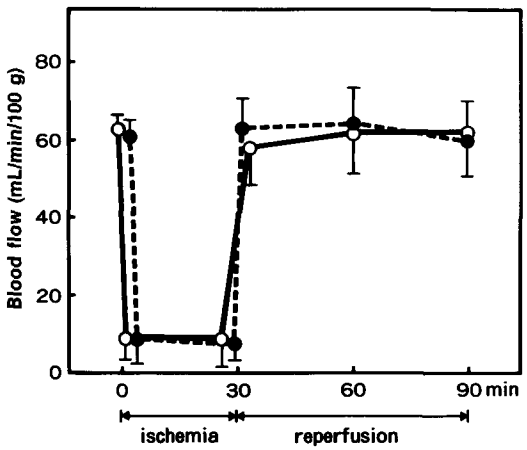

FIG. 1. Effect of CV-6209 on gastric mucosal blood flow during ischemia-reperfusion. Each point indicates the mean ± SEM of four experiments; ○—○, the group without administration of CV-6209; ●—●, the group treated with CV-2609 at a dose of 10 mg/kg.

TABLE 1

Effects of CV-6209 on Total Area of Gastric Mucosal Lesions and Thiobarbituric Acid (TBA) Reactive Substances After 30 min of Reperfusion Following 30 min of Ischemia[a]

Treatment	Total area of erosions (mm^2)	TBA reactive substances (nmol/g wet wt)
Normal	0	38.6 ± 3.0
BSA-added saline (control)	38.2 ± 5.1	62.9 ± 6.8
CV-6209 (0.1 mg/kg)	27.3 ± 4.8	55.3 ± 4.3
CV-6209 (1 mg/kg)	16.7 ± 5.2[b]	42.9 ± 3.9[b]
CV-6209 (10 mg/kg)	14.6 ± 4.2[b]	44.8 ± 3.6[b]

[a] Each value indicates the mean ± SEM of seven rats. BSA, bovine serum albumin.

[b] $P < 0.05$ when compared with the control group without administration of CV-6209. CV-6209 was injected intravenously 10 min before clamping.

ischemia-reperfusion was significantly decreased by treatment with CV-6209 at doses of 1 and 10 mg/kg (Table 1).

TBA reactive substances in the gastric mucosa significantly increased 30 min after reperfusion. This increase of TBA reactive substances was significantly inhibited by treatment with CV-6209 at doses of 1 and 10 mg/kg (Table 1). Serum TBA reactive substances in the control group and in groups treated with CV-6209 did not show any significant changes after ischemia-reperfusion when compared with a healthy group that had undergone sham operations (data not shown).

α-Tocopherol in the gastric mucosa significantly decreased after 30 min of reperfusion. This decrease was inhibited by treatment with CV-6209 at a dose of 10 mg/kg (Table 2). The serum α-tocopherol/total cholesterol ratio of the control group and of the groups treated with CV-6209 showed no significant changes after ischemia-reperfusion when compared with the healthy control group (Table 2).

TABLE 2

Effect of CV-6209 on Serum and Gastric Mucosal α-Tocopherol After 30 min Reperfusion Following 30 min Ischemia

Treatment	α-Tocopherol	
	Serum α-toc/cholesterol ratio[a] ($\times 10^{-3}$)	Gastric mucosa (ng/mg protein)
Normal	9.1 ± 0.8	13.7 ± 2.3[b]
BSA-added saline (control)	7.9 ± 0.6	9.8 ± 2.8
CV-6209 (0.1 mg/kg)	7.8 ± 1.1	10.1 ± 3.0
CV6209 (1 mg/kg)	9.0 ± 1.2	11.6 ± 2.4
CV-6209 (10 mg/kg)	8.6 ± 0.9	12.1 ± 1.8[b]

[a] Serum α-tocopherol (α-toc)/total cholesterol ratio was examined in order to eliminate the influence of serum lipids. Each value indicates the mean ± SEM of seven rats. BSA, bovine serum albumin.

[b] $P < 0.05$ when compared with the control group without administration of CV-6209. CV-6209 was injected intravenously 10 min before clamping.

DISCUSSION

The possibility that oxidative stress due to excess formation of oxygen-derived free radicals is closely involved in various disease states (17–19) has been the focus of much attention. However, it has been extremely difficult to establish a direct cause-and-effect relationship between oxygen-derived free radical formation and the actual lesions that develop. Thus, the mechanism of injury still remains unclear. However, an increasing number of reports (20,21) suggests that free radicals play an important role in ischemia-reperfusion injury based on inhibition experiments involving superoxide dismutase (SOD) and catalase. We also reported (10) that oxygen-derived free radicals and lipid peroxidation may play a role in the formation of gastric mucosal injury induced by ischemia-reperfusion, because this type of injury was significantly attenuated by SOD, catalase and other free radical scavengers. We also suggested that xanthine oxidase may be a major source of oxygen-derived free radicals in this model, because allopurinol, a competitive inhibitor of xanthine oxidase, significantly ameliorated gastric mucosal injury (22). Neutrophils also are being considered as an important source of oxygen-derived free radicals (23).

In the present study we showed that CV-6209 significantly attenuated gastric mucosal injury induced by ischemia-reperfusion and that it inhibited both an increase of TBA reactive substances, an index of lipid peroxidation, and a decrease of α-tocopherol in the gastric mucosa after ischemia-reperfusion. These results suggest the involvement of endogenous PAF in gastric mucosal injury induced by ischemia-reperfusion, because CV-6209 has been shown to act as a specific PAF receptor antagonist to compete for the PAF receptor with PAF, and to block various biological effects of PAF, with little effect on the enzymes of the arachidonic acid cascade, or on α-adrenergic receptors, on Ca channels, and so on (15). Thus, it appears that in our system the CV-6209 blocked ulcerogenic effects of endogenous PAF, which resulted in a decrease in gastric mucosal lesions. CV-6209 had no

effect on gastric mucosal blood flow during ischemia-reperfusion.

On the other hand, Yoshida (24) reported that exogenous PAF administered to rats induced acute gastric mucosal lesions, and that such lesions were attenuated by the treatment with radical scavengers or with anti-neutrophil antibody. Other authors (9,25) have reported that PAF induced superoxide generation by neutrophils in a dose-dependent manner, and that preincubation with a small amount of PAF (5×10^{-9} M) enhanced neutrophil superoxide release induced by various stimuli. We have reported (26) that CV-6209 inhibited both superoxide production by neutrophils and PAF priming *in vitro*. Considering all these reports, it appears that CV-6209 attenuated the gastric mucosal lesions mainly due to inhibition of superoxide production by neutrophils stimulated by endogenous PAF. Increased PAF levels in blood during ischemia-reperfusion have been demonstrated by others (27,28); however, we did not verify the endogenous PAF level in the present model. Such measurements would be highly informative and should be researched in the future.

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